AM100389

Avian Hepatitis E Virus, Vaccines and Methods of Protecting Against Avian Hepatitis-Splenomegaly Syndrome and Mammalian Hepatitis E

CROSS-REFERENCE TO RELATED U.S. APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119 (e) of U.S. Provisional Application No. 60/259,846, filed January 5, 2001.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The project resulting in the present invention has been supported in part by grants from the National Institutes of Health (AI01653-01, AI46505-01).

~~REFERENCE TO A "Sequence Listing"~~

Sub. B

The material on a single compact disc containing a Sequence Listing file provided in this application is incorporated by reference. The date of creation is ____, 2002 and the size is ____ bytes.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns a novel avian hepatitis E virus, immunogenic compositions, diagnostic reagents, vaccines and methods of detecting or protecting against avian hepatitis-splenomegaly syndrome and mammalian hepatitis E.

Description of the Related Art

Human hepatitis E is an important public health disease in many developing countries, and is also endemic in some industrialized countries. Hepatitis E virus (hereinafter referred to as "HEV"), the causative agent of human hepatitis E, is a single positive-stranded RNA virus without an envelope (R. H. Purcell, "Hepatitis E virus," FIELDS VIROLOGY, Vol. 2, pp. 2831-2843, B. N. Fields *et al.* eds, Lippincott-Raven Publishers, Philadelphia (3d ed. 1996)). The main route of transmission is fecal-oral, and the disease reportedly has a high mortality rate, up to 20%, in infected pregnant women. The existence of a population of individuals who are positive for HEV antibodies (anti-HEV) in industrialized countries and the recent identification of numerous genetically distinct strains of HEV have led to a hypothesis that an animal reservoir for HEV exists (X. J. Meng, "Zoonotic and xenozoonotic risks of hepatitis E virus," Infect. Dis. Rev. 2:35-41 (2000); X. J. Meng, "Novel strains of hepatitis E virus identified from humans and other animal species: Is hepatitis E a zoonosis?" J. Hepatol. 33:842-845 (2000)). In 1997, the first animal strain of HEV, swine hepatitis E virus (hereinafter referred to as "swine HEV"), was identified and characterized from a pig in the U.S. (X. J. Meng *et al.*, "A novel virus in swine is closely related to the human hepatitis E virus," Proc. Natl. Acad. Sci. USA 94:9860-9865 (1997)). Swine HEV was shown to be very closely related genetically to human HEV. Interspecies transmission of HEV has been documented: swine HEV infects non-human primates and a U.S. strain of human HEV infects pigs. These data lend further credence to the hypothesis of an animal reservoir for HEV.

Numerous genetically distinct strains of HEV have been identified from patients with acute hepatitis in both developing and industrialized countries. The two U.S. strains of human HEV recently identified from hepatitis E patients (US-1 and US-2) are genetically distinct from other known HEV strains worldwide but are closely related to each other and to the U.S. strain of swine HEV (J.C. Erker *et al.*, "A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques," J. Gen. Virol. 80:681-690 (1999); X. J. Meng *et al.*, "Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus," J. Virol. 72:9714-9721 (1998); G.G. Schlauder *et al.*, "The sequence and phylogenetic

analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States," J. Gen. Virol. 79:447-456 (1998)). Similarly, several isolates of HEV have been identified from patients in Taiwan with no history of travel to endemic region. An Italian strain of human HEV was found to share only about 79.5 to 85.8% nucleotide sequence identity with other known strains of HEV. Schlauder *et al.* recently identified another Italian and two Greek strains of HEV (G. G. Schlauder *et al.*, "Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV," J. Med. Virol. 57:243-51 (1999)). The sequences of the Greek and Italian strains of HEV differed significantly from other known strains of HEV. In endemic regions, strains of HEV, which are distinct from the previously known epidemic strains, have also been identified in Pakistan (H. Van Cuyck-Gandre *et al.*, "Short report: phylogenetically distinct hepatitis E viruses in Pakistan," Am. J. Trop. Med. Hyg. 62:187-189 (2000)), Nigeria (Y. Buisson *et al.*, "Identification of a novel hepatitis E virus in Nigeria," J. Gen. Virol. 81:903-909 (2000)) and China (Y. Wang *et al.*, "A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis," J. Gen. Virol. 80:169-77 (1999); Y. Wang *et al.*, "The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3," J. Gen. Virol. 81:1675-1686 (2000)). Six isolates of HEV were identified from Chinese hepatitis E patients that were negative for anti-HEV assayed by the serological test used (Y. Wang *et al.*, 1999, *supra*). The intriguing fact is that these recently identified strains of HEV are genetically distinct from each other and from other known strains of HEV. Although the source of these human HEV strains is not clear, it is plausible that they may be of animal origins.

Recently, several U.S. patents have issued which concern the human hepatitis E virus. U.S. Patent No. 6,022,685 describes methods and compositions for detecting anti-hepatitis E virus activity via antigenic peptides and polypeptides. U.S. Patent No. 5,885,768 discloses immunogenic peptides which are derived from the ORF1, ORF2 and ORF3 regions of hepatitis E virus, diagnostic reagents containing the peptide antigens, vaccines and immunoreactive antibodies. U.S. Patent No. 5,770,689 relates to certain ORF Z peptides of the human HEV genome. U.S. Patent No. 5,741,490 deals with a vaccine and vaccination method for preventing hepatitis E viral infections. U.S. Patent

No. 5,686,239 provides a method of detecting HEV antibodies in an individual using a peptide antigen obtained from the human HEV sequence.

Evidence of HEV infection of domestic and farm animals has been well documented (X. J. Meng, "Zoonotic and xenozoonotic risks of hepatitis E virus," Infect. Dis. Rev. 2:35-41 (2000); X. J. Meng, "Novel strains of hepatitis E virus identified from humans and other animal species: Is hepatitis E a zoonosis?" J. Hepatol., 33:842-845 (2000); R. H. Purcell, "Hepatitis E virus," FIELDS VIROLOGY, Vol. 2, pp. 2831-2843, B. N. Fields *et al.* eds, Lippincott-Raven Publishers, Philadelphia (3d ed. 1996)). Anti-HEV was detected in pigs from developing countries such as Nepal (E. T. Clayson *et al.*, "Detection of hepatitis E virus infections among domestic swine in the Kathmandu Valley of Nepal," Am. J. Trop. Med. Hyg. 53:228-232 (1995)), China (X. J. Meng *et al.*, "Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population," J. Med. Virol. 58:297-302 (1999)) and Thailand (*id.*), and from industrialized countries such as U.S. (X. J. Meng *et al.*, "A novel virus in swine is closely related to the human hepatitis E virus," Proc. Natl. Acad. Sci. USA 94:9860-9865 (1997)), Canada (X. J. Meng *et al.*, 1999, *supra*), Korea (X. J. Meng *et al.*, 1999, *id.*), Taiwan (S. Y. Hsieh *et al.*, "Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus," J. Clin. Microbiol. 37:3828-3834 (1999)), Spain (S. Pina *et al.*, "HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain," J. Hepatol. 33:826-833 (2000)) and Australia (J. D. Chandler *et al.*, "Serological evidence for swine hepatitis E virus infection in Australian pig herds," Vet. Microbiol. 68:95-105 (1999)). In addition to pigs, Kabrane-Lazizi *et al.* reported that about 77% of the rats from Maryland, 90% from Hawaii and 44% from Louisiana are positive for anti-HEV (Y. Kabrane-Lazizi *et al.*, "Evidence for wide-spread infection of wild rats with hepatitis E virus in the United States," Am. J. Trop. Med. Hyg. 61:331-335 (1999)). Favorov *et al.* also reported the detection of IgG anti-HEV among rodents in the U.S. (M.O. Favorov *et al.*, "Prevalence of antibody to hepatitis E virus among rodents in the United States," J. Infect. Dis. 181:449-455 (2000)). In Vietnam where HEV is endemic, anti-HEV was reportedly detected in 44% of chickens, 36% of pigs, 27% of dogs and 9% of rats (N. T. Tien *et al.*, "Detection of immunoglobulin G to the hepatitis E virus among several

animal species in Vietnam," Am. J. Trop. Med. Hyg. 57:211 (1997)). About 29 to 62% of cows from Somali, Tajikistan and Turkmenistan (HEV endemic regions), and about 42 to 67% of the sheep and goats from Turkmenistan and 12% of cows from Ukraine (a non-endemic region) are positive for anti-HEV (M.O. Favorov *et al.*, "Is hepatitis E an emerging zoonotic disease?" Am. J. Trop. Med. Hyg. 59:242 (1998)). Naturally acquired anti-HEV has also been reported in rhesus monkeys (S. A. Tsarev *et al.*, "Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV," J. Infect. Dis. 172:31-37 (1995)). These serological data strongly suggest that these animal species are infected with HEV or a related agent. Until recently, the source of seropositivity in these animals could not be definitively demonstrated since the virus was either not recovered from these animal species or the recovered virus was not genetically characterized to confirm its identity. The first and only animal strain of HEV that has been identified and extensively characterized thus far is swine HEV (X. J. Meng *et al.*, "A novel virus in swine is closely related to the human hepatitis E virus," Proc. Natl. Acad. Sci. USA 94:9860-9865 (1997); X. J. Meng *et al.*, "Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV," Arch. Virol. 143:1405-1415 (1998); X. J. Meng *et al.*, "Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus," J. Virol. 72:9714-9721 (1998); X. J. Meng *et al.*, "Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population," J. Med. Virol. 58:297-302 (1999)). However, because swine HEV causes only subclinical infection and mild microscopic liver lesions in pigs, it does not provide a good, adaptable animal model to study human HEV replication and pathogenesis.

Since the identification and characterization of the first animal strain of HEV (swine HEV) in the U.S. in 1997, several other HEV strains of animal origins were genetically identified. Hsieh *et al.* identified a second strain of swine HEV from a pig in Taiwan (S. Y. Hsieh *et al.*, "Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus," J. Clin. Microbiol. 37:3828-3834 (1999)). This Taiwanese strain of swine HEV shared 97.3% nucleotide sequence identity with a human strain of HEV identified from a retired

Taiwanese farmer but is genetically distinct from other known strains of HEV including the U.S. strain of swine HEV. Recently, Pina *et al.* identified a strain of HEV (E11 strain) from sewage samples of animal origin from a slaughterhouse that primarily processed pigs in Spain (S. Pina *et al.*, "HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain," J. Hepatol. 33:826-833 (2000)). The E11 strain of possible animal origin is most closely related to two Spanish strains of human HEV, and is more closely related to the U.S. swine and human strains compared to other HEV strains worldwide (*id.*). In addition to pigs, a strain of HEV was reportedly identified from tissue and fecal samples of wild-trapped rodents from Kathmandu Valley, Nepal (S. A. Tsarev *et al.*, "Naturally acquired hepatitis E virus (HEV) infection in Nepalese rodents," Am. J. Trop. Med. Hyg. 59:242 (1998)). Sequence analyses revealed that the HEV sequence recovered from Nepalese rodents is most closely related to the HEV isolates from patients in Nepal (*id.*).

Hepatitis-splenomegaly syndrome (hereinafter referred to as "HS syndrome") is an emerging disease in chickens in North America. HS syndrome in chickens was first described in 1991 in western Canada, and the disease has since been recognized in eastern Canada and the U.S. HS syndrome is characterized by increased mortality in broiler breeder hens and laying hens of 30-72 weeks of age. The highest incidence usually occurs in birds between 40 to 50 weeks of age, and the weekly mortality rate can exceed 1%. Prior to sudden death, diseased chickens usually are clinically normal, with pale combs and wattles although some birds are in poor condition. In some outbreaks, up to 20% drop in egg production was observed. Affected chickens usually show regressive ovaries, red fluid in the abdomen, and enlarged liver and spleen. The enlarged livers are mottled and stippled with red, yellow and tan foci. Similar to the microscopic lesions found in the livers of humans infected with HEV, microscopic lesions in the livers of chickens with HS syndrome vary from multifocal to extensive hepatic necrosis and hemorrhage, with infiltration of mononuclear cells around portal triads. Microscopic lesions in the spleen include lymphoid depletion and accumulation of eosinophilic materials. Numerous other names have been used to describe the disease such as necrotic hemorrhage hepatitis-splenomegaly syndrome, chronic fulminating cholangiohepatitis, necrotic hemorrhagic hepatomegalic hepatitis and hepatitis-liver hemorrhage syndrome.

The cause of HS syndrome is not known. A viral etiology for HS syndrome has been suspected but attempts to propagate the virus in cell culture or embryonated eggs were unsuccessful (J. S. Jeffrey *et al.*, "Investigation of hemorrhagic hepatosplenomegaly syndrome in broiler breeder hens," Proc. Western Poult. Dis. Conf., p. 46-48, Sacramento, CA (1998); H. L. Shivaprasad *et al.*, "Necrohemorrhagic hepatitis in broiler breeders," Proc. Western Poult. Dis. Conf., p. 6, Sacramento, CA (1995)). The pathological lesions of HS in chickens, characterized by hepatic necrosis and hemorrhage, are somewhat similar to those observed in humans infected with HEV (R. H. Purcell, "Hepatitis E virus," FIELDS VIROLOGY, Vol. 2, pp. 2831-2843, B. N. Fields *et al.* eds, Lippincott-Raven Publishers, Philadelphia (3d ed. 1996); C. Riddell, "Hepatitis-splenomegaly syndrome," DISEASE OF POULTRY, p. 1041 (1997)). Since anti-HEV was detected in 44% of chickens in Vietnam (N. T. Tien *et al.*, "Detection of immunoglobulin G to the hepatitis E virus among several animal species in Vietnam," Am. J. Trop. Med. Hyg. 57:211 (1997)), suggesting that chickens have been infected by HEV (or a related agent), it would be advantageous to find a link between HEV infection and HS syndrome in chickens. The link would permit the development of diagnostic assays and vaccines to protect against both human and chicken HEV infections thereby providing substantial public health and veterinary benefits. These goals and other desirable objectives are met by the isolation, genetic identification and characterization of the novel avian hepatitis E virus as described herein.

BRIEF SUMMARY OF THE INVENTION

The present invention concerns a novel avian hepatitis E virus, immunogenic compositions, vaccines which protect avian and mammalian species from viral infection or hepatitis-splenomegaly syndrome and methods of administering the vaccines to the avian and mammalian species to protect against viral infection or hepatitis-splenomegaly syndrome. The invention encompasses vaccines which are based on avian hepatitis E virus to protect against human hepatitis E. This invention includes methods for propagating, inactivating or attenuating hepatitis E viruses which uniquely utilize the inoculation of the live, pathogenic virus in embryonated chicken eggs. Other aspects of the present invention involve diagnostic reagents and methods for detecting the viral

causative agent and diagnosing hepatitis E in a mammal or hepatitis-splenomegaly syndrome in an avian species which employ the nucleotide sequence described herein, antibodies raised or produced against the immunogenic compositions or antigens (such as ORF2, ORF3, *etc.*) expressed in a baculovirus vector, *E. coli* and the like. The invention further embraces methods for detecting avian HEV nucleic acid sequences in an avian or mammalian species using nucleic acid hybridization probes or oligonucleotide primers for polymerase chain reaction (PCR).

BRIEF DESCRIPTION OF THE DRAWINGS

The background of the invention and its departure from the art will be further described hereinbelow with reference to the accompanying drawings, wherein:

Figure 1 shows the amplification of the 3' half of the avian HEV genome by RT-PCR: Lane M, 1 kb ladder; Lanes 1 and 2, PCR with ampli*Taq* gold polymerase; Lanes 3 and 4, PCR with ampli*Taq* gold polymerase in the presence of 5% v/v dimethyl sulfoxide (hereinafter referred to as "DMSO"); Lane 5 and 6, PCR amplification with a mixture of *Taq* polymerase and *pfu* containing in an eLONGase® Kit (GIBCO-BRL, Gaithersburg, MD).

Figures 2A and 2B represent the amino acid sequence alignment of the putative RNA-dependent RNA polymerase (RdRp) gene of avian HEV with that of known HEV strains. The conserved GDD motif is underlined. The sequence of the prototype Burmese strain is shown on top, and only differences are indicated. Deletions are indicated by hyphens (-).

Figures 3A-3C represent the sequence alignment of the ORFs 1, 2 and 3 overlapping region. The sequence of the prototype Burmese strain is shown on top, and only differences are indicated in other HEV strains. The sequence of avian HEV is shown at the bottom. The start codons are indicated by arrows, and the stop codons are indicated by three asterisks (***). The two PCR primers (FdelAHEV and RdelAHEV) used to amplify the region flanking the deletions are indicated. Deletions are indicated by hyphens (-).

Figure 4 shows the hydropathy plot of the putative ORF2 protein of avian HEV. A highly hydrophobic domain is identified at the N-terminus of the protein followed by a

hydrophilic region. The hydrophobic domain is the putative signal peptide of ORF2. The horizontal scale indicates the relative position of amino acid residues of the ORF2.

Figures 5A-5C represent the amino acid sequence alignment of the putative capsid gene (ORF2) of avian HEV with that of known HEV strains. The putative signal peptide sequence is highlighted, and the predicted cleavage site is indicated by arrowheads. The N-linked glycosylation sites are underlined in boldface. The sequence of the prototype Burmese strain is shown on top, and only differences are indicated in other HEV strains. The conserved tetrapeptide APLT is indicated (asterisks). Deletions are indicated by hyphens (-).

Sub. C3

Figure 6 illustrates the sequence alignments of the 3' noncoding region (NCR) of avian HEV with that of known HEV strains. The 3' NCR of avian HEV is shown on top, and only differences are indicated in other HEV strains. Deletions are indicated by hyphens (-).

Figure 7 represents the RT-PCR amplification of the avian HEV genomic region with a major deletion: Lane M, 1 kb ladder; Lanes 1 and 2, PCR amplification without DMSO; Lane 3, PCR amplification in the presence of 5% v/v DMSO; Lane 4, PCR amplification in the presence of 5% v/v formamide.

Figures 8A-8C provide phylogenetic trees based on the sequences of different genomic regions of HEV wherein Fig. 8A is a 439 bp sequence of the helicase gene, Fig. 8B is a 196 bp sequence of the RNA-dependent RNA polymerase gene and Fig. 8C is a 148 bp sequence of the ORF2 gene. The sequences in the three selected regions are available for most HEV strains.

Sub. C4

Figures 9A-9C represent the entire 4 kb nucleotide sequence (3931 bp plus poly(a) tract at 3' end and 5' sense primer for amplification) of the avian hepatitis E virus (which corresponds to SEQ ID NO:1).

Figure 10 represents the predicted amino acid sequence of the protein encoded by the helicase gene (which corresponds to SEQ ID NO:2).

Figure 11 represents the nucleotide sequence (439 bp) of the helicase gene (which corresponds to SEQ ID NO:3).

Figure 12 represents the predicted amino acid sequence of the protein encoded by the RdRp gene (which corresponds to SEQ ID NO:4).

Figure 13 represents the nucleotide sequence (1450 bp) of the RdRp gene (which corresponds to SEQ ID NO:5)

Figure 14 represents the predicted amino acid sequence of the protein encoded by the ORF2 gene (which corresponds to SEQ ID NO:6).

Figure 15 represents the nucleotide sequence (1821 bp) of the ORF2 gene (which corresponds to SEQ ID NO:7).

Figure 16 represents the predicted amino acid sequence of the protein encoded by the ORF3 gene (which corresponds to SEQ ID NO:8).

Figure 17 represents the nucleotide sequence (264 bp) of the ORF3 gene (which corresponds to SEQ ID NO:9).

Figure 18A (left panel) is a photograph of a normal liver from a uninoculated control SPF layer chicken. Figure 18B (right panel) is a photograph showing hepatomegaly and subcapsular hemorrhage of a liver from a SPF layer chicken experimentally infected with avian HEV. Note subcapsular hemorrhage and pronounced enlargement of right liver lobe. Liver margins are blunted indicating swelling.

Figure 19A (upper panel) shows a liver section from an uninoculated control SPF layer chicken. Note the lack of inflammatory cells anywhere in the section. Figure 19B (lower panel) shows a liver section from a SPF layer chicken experimentally infected with avian HEV (hematoxylin-eosin (HE) staining). Note the infiltration of lymphocytes in the periportal and perivascular regions.

Figure 20 illustrates a phylogenetic tree based on the helicase gene region of 9 avian HEV isolates and other selected strains of human and swine HEVs. The avian HEV isolates (shown in boldface) are all clustered with the prototype avian HEV isolate (avian HEV USA).

Figure 21A represents the expression of the C-terminal 268 amino acid sequence of truncated ORF2 capsid protein of avian HEV: Lanes 1-6, SDS-PAGE analysis of bacterial lysates at time points 0, 1, 2, 3, 4 and 6 hours after induction with IPTG; Lane 7, soluble proteins in the supernatant part of cell lysate; Lane 8, insoluble proteins after solubilization in SDS; Lane 9, SDS-PAGE analysis of 5 μg of the purified fusion protein. Figure 21B (lower panel) represents the Western blot analyses of the bacterial cell lysates at time points 0 and 3 hours after IPTG induction (Lanes 1 and 2, respectively) and of the

purified protein (Lane 3) using monoclonal antibody (MAb) against Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA) located at the N-terminal of the expressed fusion protein. The product of about 32 kD is indicated by arrows.

Figure 22A illustrates Western blot analyses of antigenic cross-reactivity of avian HEV, swine HEV, human HEV and BLSV. Purified recombinant proteins of truncated avian HEV ORF2 (Lanes 1, 6, 9, 12-15), swine HEV ORF2 (Lanes 2, 5, 8, 11, 16) and Sar-55 human HEV ORF2 (Lanes 3, 4, 7, 10, 17) were separated by SDS PAGE, transferred onto a nitrocellulose membrane and incubated with antibodies against swine HEV (Lanes 1-3), US2 human HEV (Lanes 4-6), Sar-55 human HEV (Lanes 7-9), avian HEV (Lanes 13, 16-17), and BLSV (Lane 14). Each lane contains about 250 ng of recombinant proteins. The sera were diluted 1:100 in blocking solution before added to the membranes. The development step was stopped as soon as the signal related to the preinoculation ("preimmune") sera started to appear. Preinoculation pig (Lanes 10-12) and chicken sera (Lane 15) were used as negative controls. Figures 22B and 22C present the same data in a comparative format.

Figure 23 illustrates the ELISA results generated from cross-reactivity of different antigens with different antisera and measured by optical density ("OD").

Figure 24 represents the alignment of the C-terminal 268 amino acid sequence of avian HEV with the corresponding regions of swine HEV, US2 and Sar-55 strains of human HEV. The sequence of avian HEV is shown on top. The deletions are indicated by minus (-) signs.

Figures 25A-25D show hydropathy and antigenicity plots of the truncated ORF2 proteins of avian HEV (Fig. 25A), swine HEV (Fig. 25B), Sar-55 strain of human HEV (Fig. 25C) and US2 strain of human HEV (Fig. 25D).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a novel avian hepatitis E virus (hereinafter referred to as "avian HEV"). The new animal strain of HEV, avian HEV, has been identified and genetically characterized from chickens with HS syndrome in the United States. Like swine HEV, the avian HEV identified in this invention is genetically related to human HEV strains. Unlike swine HEV that causes only

subclinical infection and mild microscopic liver lesions in pigs, avian HEV is associated with a disease (HS syndrome) in chickens. Advantageously, therefore, avian HEV infection in chickens provides a superior, viable animal model to study human HEV replication and pathogenesis.

Electron microscopy examination of bile samples of chickens with HS syndrome revealed virus-like particles. The virus was biologically amplified in embryonated chicken eggs, and a novel virus genetically related to human HEV was identified from bile samples. The 3' half of the viral genome of approximately 4 kb was amplified by reverse-transcription polymerase chain reaction (RT-PCR) and sequenced. Sequence analyses of this genomic region revealed that it contains the complete 3' noncoding region, the complete ORFs 2 and 3 genes, the complete RNA-dependent RNA polymerase (RdRp) gene and a partial helicase gene of the ORF1. The helicase gene is most conserved between avian HEV and other HEV strains, displaying 58 to 60% amino acid sequence identities.

By comparing the ORF2 sequence of avian HEV with that of known HEV strains, a major deletion of 54 amino acid residues between the putative signal peptide sequence and the conserved tetrapeptide APLT of ORF2 was identified in the avian HEV. As described herein, phylogenetic analysis indicated that avian HEV is related to known HEV strains such as the well-characterized human and swine HEV. Conserved regions of amino acid sequences exist among the ORF2 capsid proteins of avian HEV, swine HEV and human HEV. The close genetic-relatedness of avian HEV with human and swine strains of HEV suggests avian, swine and human HEV all belong to the same virus family. The avian HEV of the present invention is the most divergent strain of HEV identified thus far. This discovery has important implications for HEV animal model, nomenclature and epidemiology, and for vaccine development against chicken HS, swine hepatitis E and human hepatitis E.

Schlauder *et al.* recently reported that at least 8 different genotypes of HEV exist worldwide (G. G. Schlauder *et al.*, "Identification of 2 novel isolates of hepatitis E virus in Argentina," J. Infect. Dis. 182:294-297 (2000)). They found that the European strains (Greek 1, Greek 2, and Italy) and two Argentine isolates represent distinct genotypes. However, it is now found that the European strains (Greek 1, Greek 2 and Italy) appear to

be more related to HEV genotype 3 which consists of swine and human HEV strains from the U.S. and a swine HEV strain from New Zealand. The phylogenetic tree was based on only 148 bp sequence that is available for these strains. Additional sequence information from these strains of human HEV is required for a definitive phylogenetic analysis. HEV was classified in the family *Caliciviridae* (R. H. Purcell, "Hepatitis E virus," FIELDS VIROLOGY, Vol. 2, pp. 2831-2843, B. N. Fields *et al.* eds, Lippincott-Raven Publishers, Philadelphia (3d ed. 1996)). The lack of common features between HEV and caliciviruses has led to the recent removal of HEV from the *Caliciviridae* family, and HEV remains unclassified.

Avian HEV represents a new genotype 5. Sequence analyses revealed that the new avian HEV is genetically related to swine and human HEV, displaying 47% to 50% amino acid sequence identity in the RdRp gene, 58% to 60% identity in the helicase gene, and 42% to 44% identity in the putative capsid gene (ORF2) with the corresponding regions of known HEV strains. The genomic organization of avian HEV is very similar to that of human HEV: non-structural genes such as RdRp and helicase are located at the 5' end and structural genes (ORF2 and ORF3) are located at the 3' end of the genome. The putative capsid gene (ORF2) of avian HEV is relatively conserved at its N-terminal region (excluding the signal peptide) but is less conserved at its C-terminal region. The ORF3 gene of avian HEV is very divergent compared to that of known HEV strains. However, the C-terminus of the ORF3 of avian HEV is relatively conserved, and this region is believed to be the immuno-dominant portion of the ORF3 protein (M. Zafrullah *et al.*, "Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein," J. Virol. 73:4074-4082 (1999)). Unlike most known HEV strains, the ORF3 of avian HEV does not overlap with the ORF1. The ORF3 start codon of avian HEV is located 41 nucleotides downstream that of known HEV strains. Similar to avian HEV, the ORF3 of a strain of human HEV (HEV-T1 strain) recently identified from a patient in China does not overlap with ORF1, and its ORF3 start codon is located 28 nucleotides downstream the ORF1 stop codon (Y. Wang *et al.*, "The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3," J. Gen. Virol. 81:1675-1686 (2000)).

A major deletion was identified in the ORFs 2 and 3 overlapping region of the avian HEV genome, located between the ORF2 signal peptide and the conserved tetrapeptide APLT. It has been shown that, for certain HEV strains, this genomic region is difficult to amplify by conventional PCR methods (S. Yin *et al.*, "A new Chinese isolate of hepatitis E virus: comparison with strains recovered from different geographical regions," Virus Genes 9:23-32 (1994)), and that an addition of 5% v/v of formamide or DMSO in the PCR reaction results in the successful amplification of this genomic region. The region flanking the deletion in avian HEV genome is relatively easy to amplify by a conventional PCR modified by the method of the present invention. To rule out potential RT-PCR artifacts, the region flanking the deletion was amplified with a set of avian HEV-specific primers flanking the deletion. RT-PCR was performed with various different parameters and conditions including cDNA synthesis at 60°C, PCR amplification with higher denaturation temperature and shorter annealing time, and PCR with the addition of 5% v/v of formamide or DMSO. No additional sequence was identified, and the deletion was further verified by direct sequencing of the amplified PCR product flanking the deletion region. It is thus concluded that the observed deletion in avian HEV genome is not due to RT-PCR artifacts.

Ray *et al.* also reported a major deletion in the ORF2/ORF3 overlapping region of an Indian strain of human HEV (R. Ray *et al.*, "Indian hepatitis E virus shows a major deletion in the small open reading frame," Virology 189:359-362 (1992)). Unlike the avian HEV deletion, the deletion in the Indian strain of human HEV eliminated the ORF2 signal peptide sequence that overlaps with the ORF3. The sequence of other genomic regions of this Indian HEV strain is not available for further analysis. The biological significance of this deletion is not known. It has been shown that, when the ORF2 of a human HEV is expressed in the baculovirus system, a truncated version of ORF2 protein lacking the N-terminal 111 amino acid residues is produced. The truncated ORF2 protein was cleaved at amino acid position 111-112 (Y. Zhang *et al.*, "Expression, characterization, and immunoreactivities of a soluble hepatitis E virus putative capsid protein species expressed in insect cells," Clin. Diag. Lab. Immunol. 4:423-428 (1997)), but was still able to form virus-like particles (T. C. Li *et al.*, "Expression and self-assembly of empty virus-like particles of hepatitis E virus," J. Virol. 71:7207-7213

(1997)). Avian HEV lacks most of the N-terminal 100 amino acid residues of the ORF2, however, the conserved tetrapeptide APLT (pos. 108-111 in ORF2) and a distinct but typical signal peptide sequence are present in the ORF2 of avian HEV. Taken together, these data suggest that the genomic region between the cleavage site of the ORF2 signal peptide and the conserved tetrapeptide APLT is dispensable, and is not required for virus replication or maturation.

It has been shown that the ORF2 protein of human HEV pORF2 is the main immunogenic protein that is able to induce immune response against HEV. Recently, the C-terminal 267 amino acids of truncated ORF2 of a human HEV was expressed in a bacterial expression system showing that the sequences spanning amino acids 394 to 457 of the ORF2 capsid protein participated in the formation of strongly immunodominant epitopes on the surface of HEV particles (M. A. Riddell *et al.*, "Identification of immunodominant and conformational epitopes in the capsid protein of hepatitis E virus by using monoclonal antibodies," J. Virology 74:8011-17 (2000)). It was reported that this truncated protein was used in an ELISA to detect HEV infection in humans (D. A. Anderson *et al.*, "ELISA for IgG-class antibody to hepatitis E virus based on a highly conserved, conformational epitope expressed in Escherichia coli," J. Virol. Methods 81:131-42 (1999)). It has also been shown that C-terminus of the protein is masked when expression of the entire pORF2 is carried out in a bacterial expression system, and that the 112 amino acids located at N-terminus of ORF2 and the 50 amino acids located at the C-terminus are not involved in the formation of virus-like particles (T. C. Li *et al.*, 1997, *supra*). The expression and characterization of the C-terminal 268 amino acid residues of avian HEV ORF2 in the context of the present invention corresponds to the C-terminal 267 amino acid residues of human HEV.

The present invention demonstrates that avian HEV is antigenically related to human and swine HEVs as well as chicken BLSV. The antigenic relatedness of avian HEV ORF2 capsid protein with human HEV, swine HEV and chicken BLSV establishes that immunization with an avian HEV vaccine (either an attenuated or a recombinant vaccine) will protect not only against avian HEV infection, HS syndrome and BLSV infection in chickens but also against human and swine HEV infections in humans and swine. Thus, a vaccine based on avian HEV, its nucleic acid and the proteins encoded by

the nucleic acid will possess beneficial, broad spectrum, immunogenic activity against avian, swine and human HEVs, and BLSV.

Western blot analyses revealed that antiserum to each virus strongly reacted with homologous antigen. It was also demonstrated that the antiserum against BLSV reacted with the recombinant ORF2 protein of avian HEV, indicating that BLSV is antigenically related to avian HEV. The reaction between Sar-55 human HEV and swine HEV antigens with convalescent antiserum against avian HEV generated strong signals while the cross-reactivity of antisera with heterologous antigens was relatively weak. In ELISA, the optical densities ("ODs") obtained from the reaction of avian HEV antigen with Sar-55 HEV and swine HEV antisera were lower than the ODs obtained from the reaction of avian HEV antiserum with the HPLC-purified Sar-55 HEV and swine HEV antigens. This result may have occurred because the Sar-55 HEV and swine HEV antigens of the examples were the complete ORF2 proteins instead of the truncated avian ORF2 protein lacking the N-terminal amino acid residues.

Schofield *et al.* generated neutralizing MAbs against the capsid protein of a human HEV (D. J. Schofield *et al.*, "Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein," J. Virol. 74:5548-55 (2000)). The neutralizing MAbs recognized the linear epitope(s) located between amino acids 578 and 607. The region in avian HEV corresponding to this neutralizing epitope is located within the truncated ORF2 of avian HEV that reacted with human HEV and swine HEV anti-sera.

So far, HS syndrome has only been reported in several Provinces of Canada and a few States in the U.S. In Australia, chicken farms have been experiencing outbreaks of big liver and spleen disease (BLS) for many years. BLS was recognized in Australia in 1988 (J. H. Handlinger *et al.*, "An egg drop associated with splenomegaly in broiler breeders," Avian Dis. 32:773-778 (1988)), however, there has been no report regarding a possible link between HS in North America and BLS in Australia. A virus (designated BLSV) was isolated from chickens with BLS in Australia. BLSV was shown to be genetically related to HEV based on a short stretch of sequence available (C. J. Payne *et al.*, "Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus," Vet. Microbiol. 68:119-25 (1999)). The avian HEV

identified in this invention is closely related to BLSV identified from chickens in Australia, displaying about 80% nucleotide sequence identity in this short genomic region (439 bp). It appears that a similar virus related to HEV may have caused the HS syndrome in North American chickens and BLS in Australian chickens, but the avian HEV nevertheless remains a unique strain or isolate, a totally distinct entity from the BLS virus. Further genetic characterization of avian HEV shows that it has about 60% nucleotide sequence identities with human and swine HEVs.

In the past, the pathogenesis and replication of HEV have been poorly understood due to the absence of an efficient *in vitro* cell culture system for HEV. In this invention, it is now demonstrated that embryonated SPF chicken eggs can unexpectedly be infected with avian HEV through intravenous route (I.V.) of inoculation. Earlier studies showed that bile samples positive by EM for virus particles failed to infect embryonated chicken eggs (J. S. Jeffrey *et al.*, 1998, *supra*; H. L. Shivaprasad *et al.*, 1995, *supra*). The I.V. route of inoculation has been almost exclusively used in studies with human and swine HEV. Other inoculation routes such as the oral route have failed to infect pigs with swine HEV, even when a relatively high infectious dose ($10^{4.5}$ 50% pig infectious dose) of swine HEV was used. Based on the surprising success of the present egg inoculation experiments, it illustrates that embryonated eggs are susceptible to infection with human and avian strains of HEV making embryonated eggs a useful *in vitro* method to study HEV replication and a useful tool to manufacture vaccines that benefit public health.

The identification of avian HEV from chickens with HS in the context of this invention further strengthens the hypothesis that hepatitis E is a zoonosis. The genetic close-relatedness of avian HEV to human and swine HEV strains raises a potential public health concern for zoonosis. Recent studies showed that pig handlers are at increased risk of zoonotic HEV infection (X. J. Meng *et al.*, 1999, *supra*). Karetnyi *et al.* reported that human populations with occupational exposure to wild animals have increased risks of HEV infection (Y. V. Karetnyi *et al.*, "Hepatitis E virus infection prevalence among selected populations in Iowa," J. Clin. Virol. 14:51-55 (1999)). Since individuals such as poultry farmers or avian veterinarians may be at potential risk of zoonotic infection by avian HEV, the present invention finds broad application to prevent viral infections in humans as well as chickens and other carrier animals.

The present invention provides an isolated avian hepatitis E virus that is associated with serious viral infections and hepatitis-splenomegaly syndrome in chickens. This invention includes, but is not limited to, the virus which has a nucleotide sequence set forth in SEQ ID NO:1, its functional equivalent or complementary strand. It will be understood that the specific nucleotide sequence derived from any avian HEV will have slight variations that exist naturally between individual viruses. These variations in sequences may be seen in deletions, substitutions, insertions and the like. Thus, to distinguish the virus embraced by this invention from the Australian big liver and spleen disease virus, the avian HEV virus is characterized by having no more than about 80% nucleotide sequence homology to the BLSV.

The source of the isolated virus strain is bile, feces, serum, plasma or liver cells from chickens or human carriers suspected to have the avian hepatitis E viral infection. However, it is contemplated that recombinant DNA technology can be used to duplicate and chemically synthesize the nucleotide sequence. Therefore, the scope of the present invention encompasses the isolated polynucleotide which comprises, but is not limited to, a nucleotide sequence set forth in SEQ ID NO:1 or its complementary strand; a polynucleotide which hybridizes to and which is at least 95% complementary to the nucleotide sequence set forth in SEQ ID NO:1; or an immunogenic fragment selected from the group consisting of a nucleotide sequence in the partial helicase gene of ORF1 set forth in SEQ ID NO:3, a nucleotide sequence in the RdRp gene set forth in SEQ ID NO:5, a nucleotide sequence in the ORF2 gene set forth in SEQ ID NO:7, a nucleotide sequence in the ORF3 gene set forth in SEQ ID NO:9 or their complementary strands. The immunogenic or antigenic coding regions or fragments can be determined by techniques known in the art and then used to make monoclonal or polyclonal antibodies for immunoreactivity screening or other diagnostic purposes. The invention further encompasses the purified, immunogenic protein encoded by the isolated polynucleotides. Desirably, the protein may be an isolated or recombinant ORF2 capsid protein or an ORF3 protein.

Another important aspect of the present invention is the unique immunogenic composition comprising the isolated avian HEV or an antigenic protein encoded by an isolated polynucleotide described hereinabove and its use for raising or producing

antibodies. The composition contains a nontoxic, physiologically acceptable carrier and, optionally, one or more adjuvants. Suitable carriers, such as, for example, water, saline, ethanol, ethylene glycol, glycerol, *etc.*, are easily selected from conventional excipients and co-formulants may be added. Routine tests can be performed to ensure physical compatibility and stability of the final composition.

Vaccines and methods of using them are also included within the scope of the present invention. Inoculated avian or mammalian species are protected from serious viral infection, hepatitis-splenomegaly syndrome, hepatitis E and other related illness. The vaccines comprise, for example, an inactivated or attenuated avian hepatitis E virus, a nontoxic, physiologically acceptable carrier and, optionally, one or more adjuvants.

The adjuvant, which may be administered in conjunction with the immunogenic composition or vaccine of the present invention, is a substance that increases the immunological response when combined with the composition or vaccine. The adjuvant may be administered at the same time and at the same site as the composition or vaccine, or at a different time, for example, as a booster. Adjuvants also may advantageously be administered to the mammal in a manner or at a site different from the manner or site in which the composition or vaccine is administered. Suitable adjuvants include, but are not limited to, aluminum hydroxide (alum), immunostimulating complexes (ISCOMS), non-ionic block polymers or copolymers, cytokines (like IL-1, IL-2, IL-7, IFN-α, IFN-β, IFN-γ, *etc.*), saponins, monophosphoryl lipid A (MLA), muramyl dipeptides (MDP) and the like. Other suitable adjuvants include, for example, aluminum potassium sulfate, heat-labile or heat-stable enterotoxin isolated from *Escherichia coli*, cholera toxin or the B subunit thereof, diphtheria toxin, tetanus toxin, pertussis toxin, Freund's incomplete or complete adjuvant, *etc.* Toxin-based adjuvants, such as diphtheria toxin, tetanus toxin and pertussis toxin may be inactivated prior to use, for example, by treatment with formaldehyde.

The new vaccines of this invention are not restricted to any particular type or method of preparation. The vaccines include, but are not limited to, modified live vaccines, inactivated vaccines, subunit vaccines, attenuated vaccines, genetically engineered vaccines, *etc.* These vaccines are prepared by general methods known in the art modified by the new use of embryonated eggs. For instance, a modified live vaccine

may be prepared by optimizing avian HEV propagation in embryonated eggs as described herein and further virus production by methods known in the art. Since avian HEV cannot grow in the standard cell culture, the avian HEV of the present invention can uniquely be attenuated by serial passage in embryonated chicken eggs. The virus propagated in eggs may be lyophilized (freeze-dried) by methods known in the art to enhance preservability for storage. After subsequent rehydration, the material is then used as a live vaccine.

The advantages of live vaccines is that all possible immune responses are activated in the recipient of the vaccine, including systemic, local, humoral and cell-mediated immune responses. The disadvantages of live virus vaccines, which may outweigh the advantages, lie in the potential for contamination with live adventitious viral agents or the risk that the virus may revert to virulence in the field.

To prepare inactivated virus vaccines, for instance, the virus propagation and virus production in embryonated eggs are again first optimized by methods described herein. Serial virus inactivation is then optimized by protocols generally known to those of ordinary skill in the art or, preferably, by the methods described herein.

Inactivated virus vaccines may be prepared by treating the avian HEV with inactivating agents such as formalin or hydrophobic solvents, acids, *etc.*, by irradiation with ultraviolet light or X-rays, by heating, *etc.* Inactivation is conducted in a manner understood in the art. For example, in chemical inactivation, a suitable virus sample or serum sample containing the virus is treated for a sufficient length of time with a sufficient amount or concentration of inactivating agent at a sufficiently high (or low, depending on the inactivating agent) temperature or pH to inactivate the virus. Inactivation by heating is conducted at a temperature and for a length of time sufficient to inactivate the virus. Inactivation by irradiation is conducted using a wavelength of light or other energy source for a length of time sufficient to inactivate the virus. The virus is considered inactivated if it is unable to infect a cell susceptible to infection.

The preparation of subunit vaccines typically differs from the preparation of a modified live vaccine or an inactivated vaccine. Prior to preparation of a subunit vaccine, the protective or antigenic components of the vaccine must be identified. Such protective or antigenic components include certain amino acid segments or fragments of the viral

capsid proteins which raise a particularly strong protective or immunological response in chickens; single or multiple viral capsid proteins themselves, oligomers thereof, and higher-order associations of the viral capsid proteins which form virus substructures or identifiable parts or units of such substructures; oligoglycosides, glycolipids or glycoproteins present on or near the surface of the virus or in viral substructures such as the lipoproteins or lipid groups associated with the virus, *etc.* Preferably, the capsid protein (ORF2) is employed as the antigenic component of the subunit vaccine. Other proteins may also be used such as those encoded by the nucleotide sequence in the ORF3 gene. These immunogenic components are readily identified by methods known in the art. Once identified, the protective or antigenic portions of the virus (*i.e.*, the "subunit") are subsequently purified and/or cloned by procedures known in the art. The subunit vaccine provides an advantage over other vaccines based on the live virus since the subunit, such as highly purified subunits of the virus, is less toxic than the whole virus.

If the subunit vaccine is produced through recombinant genetic techniques, expression of the cloned subunit such as the ORF2 (capsid) and ORF3 genes, for example, may be optimized by methods known to those in the art (see, for example, Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, MA. (1989)). On the other hand, if the subunit being employed represents an intact structural feature of the virus, such as an entire capsid protein, the procedure for its isolation from the virus must then be optimized. In either case, after optimization of the inactivation protocol, the subunit purification protocol may be optimized prior to manufacture.

To prepare attenuated vaccines, the live, pathogenic virus is first attenuated (rendered nonpathogenic or harmless) by methods known in the art or, preferably, as described herein. For instance, attenuated viruses may be prepared by the technique of the present invention which involves the novel serial passage through embryonated chicken eggs. Attenuated viruses can be found in nature and may have naturally-occurring gene deletions or, alternatively, the pathogenic viruses can be attenuated by making gene deletions or producing gene mutations. The attenuated and inactivated virus vaccines comprise the preferred vaccines of the present invention.

Genetically engineered vaccines, which are also desirable in the present invention, are produced by techniques known in the art. Such techniques involve, but are not limited to, the use of RNA, recombinant DNA, recombinant proteins, live viruses and the like.

For instance, after purification, the wild-type virus may be isolated from suitable clinical, biological samples such as feces or bile by methods known in the art, preferably by the method taught herein using embryonated chicken eggs as hosts. The RNA is extracted from the biologically pure virus or infectious agent by methods known in the art, preferably by the guanidine isothiocyanate method using a commercially available RNA isolation kit (for example, the kit available from Statagene, La Jolla, CA.) and purified by methods known in the art, preferably by ultracentrifugation in a CsCl gradient. RNA may be further purified or enriched by oligo(dT)-cellulose column chromatography. The cDNA of viral genome is cloned into a suitable host by methods known in the art (see Maniatis *et al.*, *id.*), and the virus genome is then analyzed to determine essential regions of the genome for producing antigenic portions of the virus. Thereafter, the procedure is generally the same as that for the modified live vaccine, an inactivated vaccine or a subunit vaccine.

Genetically engineered vaccines based on recombinant DNA technology are made, for instance, by identifying the portion of the viral gene which encodes for proteins responsible for inducing a stronger immune or protective response in chickens (*e.g.*, proteins derived from ORF1, ORF2, ORF3, *etc.*). Such identified genes or immuno-dominant fragments can be cloned into standard protein expression vectors, such as the baculovirus vector, and used to infect appropriate host cells (see, for example, O'Reilly *et al.*, "Baculovirus Expression Vectors: A Lab Manual," Freeman & Co. (1992)). The host cells are cultured, thus expressing the desired vaccine proteins, which can be purified to the desired extent and formulated into a suitable vaccine product.

Genetically engineered proteins, useful in vaccines, for instance, may be expressed in insect cells, yeast cells or mammalian cells. The genetically engineered proteins, which may be purified or isolated by conventional methods, can be directly inoculated into an avian or mammalian species to confer protection against avian or human hepatitis E.

An insect cell line (like HI-FIVE) can be transformed with a transfer vector containing polynucleic acids obtained from the virus or copied from the viral genome which encodes one or more of the immuno-dominant proteins of the virus. The transfer vector includes, for example, linearized baculovirus DNA and a plasmid containing the desired polynucleotides. The host cell line may be co-transfected with the linearized baculovirus DNA and a plasmid in order to make a recombinant baculovirus.

Alternatively, RNA or DNA from the HS infected carrier or the isolated avian HEV which encode one or more capsid proteins can be inserted into live vectors, such as a poxvirus or an adenovirus and used as a vaccine.

An immunologically effective amount of the vaccine of the present invention is administered to an avian or mammalian species in need of protection against said infection or syndrome. The "immunologically effective amount" can be easily determined or readily titrated by routine testing. An effective amount is one in which a sufficient immunological response to the vaccine is attained to protect the bird or mammal exposed to the virus which causes chicken HS, human hepatitis E, swine hepatitis E or related illness. Preferably, the avian or mammalian species is protected to an extent in which one to all of the adverse physiological symptoms or effects of the viral disease are found to be significantly reduced, ameliorated or totally prevented.

The vaccine can be administered in a single dose or in repeated doses. Dosages may contain, for example, from 1 to 1,000 micrograms of virus-based antigen (dependent upon the concentration of the immuno-active component of the vaccine), but should not contain an amount of virus-based antigen sufficient to result in an adverse reaction or physiological symptoms of viral infection. Methods are known in the art for determining or titrating suitable dosages of active antigenic agent based on the weight of the bird or mammal, concentration of the antigen and other typical factors.

The vaccine can be administered to chickens, turkeys or other farm animals in close contact with chickens, for example, pigs. Also, the vaccine can be given to humans such as chicken or poultry farmers who are at high risk of being infected by the viral agent. It is contemplated that a vaccine based on the avian HEV can be designed to provide broad protection against both avian and human hepatitis E. In other words, the vaccine based on the avian HEV can be preferentially designed to protect against human

hepatitis E through the so-called "Jennerian approach" (*i.e.*, cowpox virus vaccine can be used against human smallpox by Edward Jenner). Desirably, the vaccine is administered directly to an avian or mammalian species not yet exposed to the virus which causes HS, hepatitis E or related illness. The vaccine can conveniently be administered orally, intrabuccally, intranasally, transdermally, parenterally, *etc*. The parenteral route of administration includes, but is not limited to, intramuscular, intravenous, intraperitoneal and subcutaneous routes.

When administered as a liquid, the present vaccine may be prepared in the form of an aqueous solution, a syrup, an elixir, a tincture and the like. Such formulations are known in the art and are typically prepared by dissolution of the antigen and other typical additives in the appropriate carrier or solvent systems. Suitable carriers or solvents include, but are not limited to, water, saline, ethanol, ethylene glycol, glycerol, *etc.* Typical additives are, for example, certified dyes, flavors, sweeteners and antimicrobial preservatives such as thimerosal (sodium ethylmercurithiosalicylate). Such solutions may be stabilized, for example, by addition of partially hydrolyzed gelatin, sorbitol or cell culture medium, and may be buffered by conventional methods using reagents known in the art, such as sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate, potassium dihydrogen phosphate, a mixture thereof, and the like.

Liquid formulations also may include suspensions and emulsions which contain suspending or emulsifying agents in combination with other standard co-formulants. These types of liquid formulations may be prepared by conventional methods. Suspensions, for example, may be prepared using a colloid mill. Emulsions, for example, may be prepared using a homogenizer.

Parenteral formulations, designed for injection into body fluid systems, require proper isotonicity and pH buffering to the corresponding levels of mammalian body fluids. Isotonicity can be appropriately adjusted with sodium chloride and other salts as needed. Suitable solvents, such as ethanol or propylene glycol, can be used to increase the solubility of the ingredients in the formulation and the stability of the liquid preparation. Further additives which can be employed in the present vaccine include, but are not limited to, dextrose, conventional antioxidants and conventional chelating agents

such as ethylenediamine tetraacetic acid (EDTA). Parenteral dosage forms must also be sterilized prior to use.

Also included within the scope of the present invention is a novel method for propagating, inactivating or attenuating the pathogenic hepatitis E virus (avian, swine, human, *etc.*) which comprises inoculating an embryonated chicken egg with a live, pathogenic hepatitis E virus contained in a biological sample from bile, feces, serum, plasma, liver cell, *etc.*, preferably by intravenous injection, and either recovering a live, pathogenic virus for further research and vaccine development or continuing to pass the pathogenic virus serially through additional embryonated chicken eggs until the pathogenic virus is rendered inactivated or attenuated. Propagating live viruses through embryonated chicken eggs according to the present invention is a unique method which others have failed to attain. Vaccines are typically made by serial passage through cell cultures but avian HEV, for example, cannot be propagated in conventional cell cultures. Using embryonated chicken eggs provides a novel, viable means for inactivating or attenuating the pathogenic virus in order to be able to make a vaccine product. The inactivated or attenuated strain, which was previously unobtainable, can now be incorporated into conventional vehicles for delivering vaccines.

Additionally, the present invention provides a useful diagnostic reagent for detecting the avian or mammalian HEV infection or diagnosing hepatitis-splenomegaly syndrome in an avian or mammalian species which comprise a monoclonal or polyclonal antibody purified from a natural host such as, for example, by inoculating a chicken with the avian HEV or the immunogenic composition of the invention in an effective immunogenic quantity to produce a viral infection and recovering the antibody from the serum of the infected chicken. Alternatively, the antibodies can be raised in experimental animals against the natural or synthetic polypeptides derived or expressed from the amino acid sequences or immunogenic fragments encoded by the nucleotide sequence of the isolated avian HEV. For example, monoclonal antibodies can be produced from hybridoma cells which are obtained from mice such as, for example, Balb/c, immunized with a polypeptide antigen derived from the nucleotide sequence of the isolated avian HEV. Selection of the hybridoma cells is made by growth in hyproxanthine, thymidine and aminopterin in a standard cell culture medium like Dulbecco's modified Eagle's

medium (DMEM) or minimal essential medium. The hybridoma cells which produce antibodies can be cloned according to procedures known in the art. Then, the discrete colonies which are formed can be transferred into separate wells of culture plates for cultivation in a suitable culture medium. Identification of antibody secreting cells is done by conventional screening methods with the appropriate antigen or immunogen. Cultivating the hybridoma cells *in vitro* or *in vivo* by obtaining ascites fluid in mice after injecting the hybridoma produces the desired monoclonal antibody via well-known techniques.

For another alternative method, avian HEV capsid protein can be expressed in a baculovirus expression system or *E. coli* according to procedures known in the art. The expressed recombinant avian HEV capsid protein can be used as the antigen for diagnosis of HS or human hepatitis E in an enzyme-linked immunoabsorbent Assay (ELISA). The ELISA assay based on the avian recombinant capsid antigen, for example, can be used to detect antibodies to avian HEV in avian and mammalian species. Although the ELISA assay is preferred, other known diagnostic tests can be employed such as immunofluorescence assay (IFA), immunoperoxidase assay (IPA), *etc.*

Desirably, a commercial ELISA diagnostic assay in accordance with the present invention can be used to diagnose avian HEV infection and HS syndrome in chickens. The examples illustrate using purified ORF2 protein of avian HEV to develop an ELISA assay to detect anti-HEV in chickens. Weekly sera collected from SPF chickens experimentally infected with avian HEV, and negative sera from control chickens are used to validate the assay. This ELISA assay has been successfully used in the chicken studies to monitor the course of seroconversion to anti-HEV in chickens experimentally infected with avian HEV. Further standardization of the test by techniques known to those skilled in the art may optimize the commercialization of a diagnostic assay for avian HEV. Other diagnostic assays can also be developed as a result of the findings of the present invention such as a nucleic acid-based diagnostic assay, for example, an RT-PCR assay and the like. Based on the description of the sequences of the partial genomes of the nine new strains of avian HEV, the RT-PCR assay and other nucleic acid-based assays can be standardized to detect avian HEV in clinical samples.

The antigenic cross-reactivity of the truncated ORF2 capsid protein (pORF2) of avian HEV with swine HEV, human HEV and the chicken big liver and spleen disease virus (BLSV) is shown in the below examples. The sequence of C-terminal 268 amino acid residuals of avian HEV ORF2 was cloned into expression vector pRSET-C and expressed in *Escherichia coli* (*E. coli*) strain BL21(DE3)pLysS. The truncated ORF2 protein was expressed as a fusion protein and purified by affinity chromatography. Western blot analysis revealed that the purified avian HEV ORF2 protein reacted with the antisera raised against the capsid protein of Sar-55 human HEV and with convalescent antisera against swine HEV and US2 human HEV as well as antiserum against BLSV. The antiserum against avian HEV also reacted with the HPLC-purified recombinant capsid proteins of swine HEV and Sar-55 human HEV. The antiserum against US2 strain of human HEV also reacted with recombinant ORF2 proteins of both swine HEV and Sar-55 human HEV. Using ELISA further confirmed the cross reactivity of avian HEV putative capsid protein with the corresponding genes of swine HEV and human HEVs. The results show that avian HEV shares some antigenic epitopes in its capsid protein with swine and human HEVs as well as BLSV, and establish the usefulness of the diagnostic reagents for HEV diagnosis as described herein.

The diagnostic reagent is employed in a method of the invention for detecting the avian or mammalian hepatitis E viral infection or diagnosing hepatitis-splenomegaly syndrome in an avian or mammalian species which comprises contacting a biological sample of the bird or mammal with the aforesaid diagnostic reagent and detecting the presence of an antigen-antibody complex by conventional means known to those of ordinary skill in the art. The biological sample includes, but is not limited to, blood, plasma, bile, feces, serum, liver cell, *etc.* To detect the antigen-antibody complex, a form of labeling is often used. Suitable radioactive or non-radioactive labeling substances include, but are not limited to, radioactive isotopes, fluorescent compounds, dyes, *etc.* The detection or diagnosis method of this invention includes immunoassays, immunometric assays and the like. The method employing the diagnostic reagent may also be accomplished in an *in vitro* assay in which the antigen-antibody complex is detected by observing a resulting precipitation. The biological sample can be utilized from any avian species such as chickens, turkeys, *etc.* or mammals such as pigs and other

farm animals or humans, in particular, chicken farmers who have close contact with chickens. If the bird or the mammal is suspected of harboring a hepatitis E viral infection and exhibiting symptoms typical of hepatitis-splenomegaly syndrome or other related illness, the diagnostic assay will be helpful to determine the appropriate course of treatment once the viral causative agent has been identified.

Another preferred embodiment of the present invention involves methods for detecting avian HEV nucleic acid sequences in an avian or mammalian species using nucleic acid hybridization probes or oligonucleotide primers for polymerase chain reaction (PCR) to further aid in the diagnosis of viral infection or disease. The diagnostic tests, which are useful in detecting the presence or absence of the avian hepatitis E viral nucleic acid sequence in the avian or mammalian species, comprise, but are not limited to, isolating nucleic acid from the bird or mammal and then hybridizing the isolated nucleic acid with a suitable nucleic acid probe or probes, which can be radio-labeled, or a pair of oligonucleotide primers derived from the nucleotide sequence set forth in SEQ ID NO:1 and determining the presence or absence of a hybridized probe complex. Conventional nucleic acid hybridization assays can be employed by those of ordinary skill in this art. For example, the sample nucleic acid can be immobilized on paper, beads or plastic surfaces, with or without employing capture probes; an excess amount of radio-labeled probes that are complementary to the sequence of the sample nucleic acid is added; the mixture is hybridized under suitable standard or stringent conditions; the unhybridized probe or probes are removed; and then an analysis is made to detect the presence of the hybridized probe complex, that is, the probes which are bound to the immobilized sample. When the oligonucleotide primers are used, the isolated nucleic acid may be further amplified in a polymerase chain reaction or other comparable manner before analysis for the presence or absence of the hybridized probe complex. Preferably, the polymerase chain reaction is performed with the addition of 5% v/v of formamide or dimethyl sulfoxide.

The following examples demonstrate certain aspects of the present invention. However, it is to be understood that these examples are for illustration only and do not purport to be wholly definitive as to conditions and scope of this invention. It should be appreciated that when typical reaction conditions (*e.g.*, temperature, reaction times, *etc.*)

have been given, the conditions both above and below the specified ranges can also be used, though generally less conveniently. The examples are conducted at room temperature (about 23°C to about 28°C) and at atmospheric pressure. All parts and percents referred to herein are on a weight basis and all temperatures are expressed in degrees centigrade unless otherwise specified.

A further understanding of the invention may be obtained from the non-limiting examples that follow below.

EXAMPLE 1

Biological Amplification of the Virus in Embryonated Chicken Eggs

A sample of bile collected from a chicken with HS in California was used in this study. Electron microscopy (EM) examination showed that this bile sample was positive for virus particles of 30 to 40 nm in diameter. The limited bile materials containing the virus prevented the performance of extensive genetic identification and characterization of the virus. A preliminary study was conducted to determine if the virus could be biologically amplified in embryonated chicken eggs. SPF eggs were purchased at one day of age (Charles River SPAFAS, Inc., North Franklin, CT.) and incubated for 9 days in a 37°C egg incubator. At 9 days of embryonated age, 6 eggs were inoculated intravenously with 100 µl of a 10^{-3} dilution and 6 eggs with a 10^{-4} dilution in phosphate buffered saline (PBS) of the positive bile sample. Six eggs were uninoculated as controls. The inoculated eggs were incubated at 37°C until 21 days of age (before natural hatching), at which time the embryos were sacrificed. Bile and liver samples were collected and tested by RT-PCR for evidence of virus replication. The virus recovered from infected eggs was used as the virus source for further characterization.

EXAMPLE 2

Amplification of the 3' Half of the Viral Genome

Based on the assumption that the putative virus associated with HS in chickens shared nucleotide sequence similarity with human and swine HEV, a modified 3' RACE (Rapid Amplification of cDNA Ends) system was employed to amplify the 3'-half of the viral genome. Briefly, the sense primer, F4AHEV (Table 1 below), was chosen from a

conserved region in ORF1 among known swine and human HEV strains including the big liver and spleen disease virus (BLSV) identified from chickens in Australia (C. J. Payne *et al.*, "Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus," Vet. Microbiol. 68:119-25 (1999)). The antisense primers included two anchored commercial primers of nonviral origin (GIBCO-BRL, Gaithersburg, MD): AUAP (Abridged Universal Amplification Primer) and AP (Adapter Primer) with a poly (T) stretch (Table 1, below). Total RNA was extracted from 100 µl of the bile by TriZol reagent (GIBCO-BRL), and resuspended in 11.5 µl of DNase-, RNase- and proteinase-free water (Eppendorf Scientific, Inc., now Brinkmann Instruments, Inc., Westbury, NY). Total RNA was reverse-transcribed at 42°C for 90 minutes in the presence of reverse transcription reaction mixtures consisting of 11.5 µl of the total RNA, 1 µl of Superscript II reverse transcriptase (GIBCO-BRL), 1 µl of 10 µM antisense primer, 0.5 µl of RNase inhibitor (GIBCO-BRL), 0.5 µl of dithioteritol, and 4 µl of 5 X RT buffer.

PCR was performed with a mixture of a *Taq* DNA polymerase and a proofreading *pfu* polymerase contained in an eLONGase® Kit (GIBCO-BRL, Gaithersburg, MD). The PCR reaction was carried out according to the instructions supplied with the kit and consisted of 10 µl of cDNA, 1.7 mM $MgCL_2$ and 1 µl of each 10 µM sense and antisense primers. Alternatively, Ampli*Taq* gold polymerase (Perkin-Elmer, Wellesley, MA) with and without 5% v/v dimethyl sulfoxide (DMSO) was used. The PCR reaction consisted of a denaturation at 94°C for 1 minute, followed by 5 cycles of denaturation at 94°C for 40 seconds, annealing at 42°C for 40 seconds, extension at 68°C for 5 minutes, 16 cycles of a touch down PCR with the starting annealing temperature at 59°C which was reduced by 1 degree every 2 cycles, and then 11 cycles of amplification with an annealing temperature at 51°C, followed by a final extension at 74°C for 10 minutes. The resulting PCR product was analyzed on a 0.8% w/v agarose gel. When Ampli*Taq* gold polymerase was used, the thermal cycle profile and parameters remained the same except that the enzyme was first activated by incubation at 95°C for 9 minutes.

Table 1. Synthetic oligonucleotide primers used for PCR amplification and DNA sequencing of the avian HEV genome

Primer Designation	Nucleotide Sequence (5' to 3')	Position[a]
Sequencing Primers	TTGGTGGGGTGCTGGTCGAGATTG	438-415
	CCGGGAGCGCTGTAGTGTGATTGATGT	389-414
	CCATAAATCCACCCGGGCCTGT	649-628
	CAATCAACCCCTCAACACTGGA	871-892
	ATTTACGCTGGATGACCCTGTTGCAC	1038-1013
	GGATGCCCGATTGGATGGTAGCCTT	1306-1330
	GGATGCCCGATTGGATGGTAGCCTT	1330-1306
	TCCCGGGAGCTGGTGTTGTCTGC	1633-1655
	GATGCCCGATTGGATGGTAGCCTTGTA	1307-1333
	CTGACAAGAACTGGGGGCCCGACAT	1708-1644
	CAATGTGCTGCGGGGTGTCAAG	2046-2067
	AATGTGCTGCGGGGTGTCAAGGG	2069-2047
	CAAATGCGGTGGGCGGCTTCTCTATA	2470-2445
	ACTGCTGAGGATGGCGAAATTGGTC	2945-2921
	ACCGACATATACAGTTTCACCTCAG	3072-3096
	CTGAGGTGAAACTGTATATGTCGGT	3096-3072
	GAACGGCGAGCCTGAGGTGAAACTGT	3061-3086
	TTCTCTATAAGCATGGCCTATTG	2486-2462
	CAGAATGGTAGCTCCGTGGTTTGGTATGC	3603-3575
	TCTTCAGAATGGTAGCTCCGTGGTTTG	3571-3597
F4AHEV	GCTAGGCGACCCGCACCAGAT	non-viral (GIBCO)
AP	GACTCGAGTCGACATCGA$(T)_{17}$	non-viral (GIBCO)
PAUP	GACTCGAGTCGACATCGA	non-viral (GIBCO)
FdelAHEV	GGGGCCCGACATTCAGCGGATGCAG	1666-1690
RdelAHEV	GCCGCGGTGACAACGTCTGTGAGAGG	2143-2168

[a] The positions are relative to the 3931 bp sequence of avian HEV determined in the present invention.

EXAMPLE 3

Cloning of the Amplified PCR Product

A PCR product of approximately 4 kb was amplified by the modified 3' RACE system. The PCR product was excised and eluted from the agarose gel with the CoNcERT™ Rapid Gel Extraction System (GIBCO-BRL). The purified PCR product was subsequently cloned into a TA vector. The recombinant plasmid was used to transform competent cells supplied in the AdvanTAge™ PCR Cloning Kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instruction. White colonies were selected and grown in LB broth containing 100 μg/ml of ampicillin. The recombinant plasmids containing the insert were isolated with a Plasmid DNA Isolation kit (Qiagen Inc., Valencia, CA).

EXAMPLE 4

DNA Sequencing

Three independent cDNA clones containing the approximately 4 kb insert were selected and sequenced at Virginia Tech DNA Sequencing Facility with an Automated DNA Sequencer (Applied Biosystem, Inc., Foster City, CA). Primer walking strategy was employed to determine the nucleotide sequence of both DNA strands of the three independent cDNA clones. The M13 forward and reverse primers as well as sixteen avian HEV specific primers (Table 1, above) were used to determine the nucleotide sequence of the approximately 4 kb viral genome. To facilitate DNA sequencing, a unique EcoR I restriction site that is present in this 4 kb viral genomic fragment was utilized. The recombinant plasmid with the 4 kb insert was digested by the EcoR I restriction enzyme, and the resulting two EcoR I fragments were subcloned into pGEM-9zf (-) (Promega, Madison, WI). The cDNA subclones were also used to determine the sequence by primer walking strategy. The sequence at the 5' end of the fragment was further confirmed by direct sequencing of the PCR product amplified with avian HEV-specific primers.

EXAMPLE 5

Sequence and Phylogenetic Analyses

The complete sequence of the approximately 4 kb viral genomic fragment was assembled and analyzed with the MacVector® (Oxford Molecular, Inc., Madison, WI) and DNAstar (DNASTAR, Inc., Madison, WI) computer programs. For any given region, the consensus sequence was derived from at least three independent cDNA clones. The putative signal peptide of the ORF2 protein was predicted with the SignalP V1.1 program (http://www.cbs.dtu.dk/services/SignalP). The hydrophobicity analysis of the putative ORF2 protein was performed with the MacVector program using Sweet/Eisenberg method (R. M. Sweet *et al.*, "Correlation of sequence hydrophobicities measures similarity in three-dimensional protein structure," J. Mol. Biol. 171:479-488 (1983)). Phylogenetic analyses were conducted with the aid of the PAUP program (David L. Swofford, Smithsonian Institution, Washington, D.C., and distributed by Sinauer Associates, Inc., Sunderland, Mass).

For most HEV strains, the sequences are available only in certain genomic regions. Therefore, to better understand the phylogenetic relationship of known HEV strains, phylogenetic analyses were based on three different genomic regions: a 148 bp fragment of the ORF2 gene in which the sequences of most HEV strains are available, a 196 bp fragment of the RdRp gene, and a 439 bp fragment of the helicase gene in which the sequence of BLSV is known. Phylogenetic analyses were also performed with the complete RdRp and ORF2 genes from known HEV strains. The branch-and-bound and midpoint rooting options were used to produce the phylogenetic trees. The sequences of known HEV strains used in the sequence and phylogenetic analyses were either published or available in the Genbank database: Nepal (V. Gouvea *et al.*, "Hepatitis E virus in Nepal: similarities with the Burmese and Indian variants," Virus Res. 52:87-96 (1997)), Egypt 93 (S. A. Tsarev *et al.*, "Phylogenetic analysis of hepatitis E virus isolates from Egypt," J. Med. Virol . 57:68-74 (1999)), Egypt 94 (*id.*), Morroco (J. Meng *et al.*, "Primary structure of open reading frame 2 and 3 of the hepatitis E virus isolated from Morocco," J. Med. Virol. 57:126-133 (1999)), Pakistan (strain Sar55) (S. A. Tsarev *et al.*, "Characterization of a prototype strain of hepatitis E virus," Proc. Natl. Acad. Sci. U S A. 89:559-63 (1992)), Burma (G. R. Reyes *et al.*, "Isolation of a cDNA from the virus

responsible for enterically transmitted non-A, non-B hepatitis," Science 247:1335-1339 (1990)), Myanmar (A.W. Tam *et al.*, "Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome," Virology 185:120-131 (1991)), Vietnam (accession no. AF 170450), Greek 1 (G. G. Schlauder *et al.*, "Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV," J. Med. Virol. 57:243-51 (1999)), Greek 2 (*id.*), Italy (*id.*), Mexico (C. C. Huang *et al.*, "Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV)," Virology 191:550-558 (1992)), US1 (G.G. Schlauder *et al.*, "The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States," J. Gen. Virol. 79:447-456 (1998)), US2 (J.C. Erker *et al.*, "A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques," J. Gen. Virol. 80:681-690 (1999)), the U.S. strain of swine HEV (X. J. Meng *et al.*, "A novel virus in swine is closely related to the human hepatitis E virus," Proc. Natl. Acad. Sci. USA 94:9860-9865 (1997); X. J. Meng *et al.*, "Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus," J. Virol. 72:9714-9721 (1998)), the New Zealand strain of swine HEV (accession no. AF200704), Indian strains including Hyderabad (S. K. Panda *et al.*, "The *in vitro*-synthesized RNA from a cDNA clone of hepatitis E virus is infectious," J. Virol. 74:2430-2437 (2000)), Madras (accession no. X99441), X98292 (strain HEV037) (M.C. Donati *et al.*, "Sequence analysis of full-length HEV clones derived directly from human liver in fulminant hepatitis E," VIRAL HEPATITIS AND LIVER DISEASE, pp. 313-316 (M. Rizzetto *et al.*, eds., Edizioni Minerva Medica, Torino, 1997)), AKL 90 (V. A. Arankalle *et al.*, "Phylogenetic analysis of hepatitis E virus isolates from India (1976-1993)," J. Gen. Virol. 80:1691-1700 (1999)), and U22532 (S. K. Panda *et al.*, "An Indian strain of hepatitis E virus (HEV): cloning, sequence, and expression of structural region and antibody responses in sera from individuals from an area of high-level HEV endemicity," J. Clin. Microbiol. 33:2653-2659 (1995)), Taiwanese strains including TW4E, TW7E and TW8E, and Chinese strains including 93G (accession no. AF145208), L25547, Hetian, KS2, D11093 (strain Uigh 179), D11092, HEV-T1, Ch-T11 (accession no. AF151962) and Ch-T21 (accession no. AF151963).

EXAMPLE 6

Propagation of Avian HEV in Embryonated Chicken Eggs

The aim of this preliminary experiment was to generate, by biological amplification of the virus in embryonated eggs, sufficient amounts of virus for further studies, and to determine if avian HEV replicates in eggs. The undiluted positive bile sample contained about 10^7 genomic equivalents (GE) of avian HEV per ml of bile. Embryonated SPF chicken eggs were intravenously inoculated with a diluted bile sample containing avian HEV. Five out of six embryos inoculated with 10^{-3} dilution and three out of six embryos inoculated with 10^{-4} dilution died before 21 days of embryonated age. At 12 days postinoculation (21 days of embryonated age), the remaining 4 inoculated embryos were sacrificed. The inoculated embryos showed congestion of yolk sac and hemorrhage in the liver. There are no apparent gross lesions in uninoculated embryos. Samples of bile and liver collected from inoculated eggs at the day of natural hatching (12 days postinoculation) were tested by RT-PCR. Avian HEV RNA was detected in both bile and liver samples. The titer of virus in the bile recovered from embryos was about 10^7 genomic equivalent per ml (GE/ml), indicating that avian HEV replicates in embryonated chicken eggs. The virus recovered from inoculated eggs was used as the source for subsequent genetic characterization.

EXAMPLE 7

Amplification and Sequence Determination of the 3' Half of the Avian HEV Genome

An attempt to amplify an approximately 4 kb fragment at the 3' half of the avian HEV genome was pursued. The attempt initially failed to amplify the fragment with AmpliTaq Gold polymerase. However, in the presence of 5% v/v DMSO, a weak signal of a PCR product of approximately 4 kb was generated with AmpliTaq Gold polymerase (Fig. 1). To increase the amplification efficiency, the PCR with a mixture of *pfu* polymerase and *Taq* DNA polymerase was performed in the presence of 10 µl of cDNA and 1.7 mM $MgCL_2$ by using an eLONGase® kit. After 32 cycles of amplification, an abundant amount of PCR product of approximately 4 kb was generated (Fig. 1). The resulting PCR product was subsequently cloned into a TA vector. Three cDNA clones

were selected and sequenced for both DNA strands. The number of poly (A) residues at the 3' end of each of the three cDNA clones was different (19, 23, and 26 residues, respectively), indicating that these 3 clones sequenced represent independent cDNA clones. This 4 kb genomic fragment contains the complete ORFs 2 and 3 (set forth in SEQ ID NO:7 and SEQ ID NO:9, respectively), the complete RNA-dependent RNA polymerase (RdRp) gene (set forth in SEQ ID NO:5), a partial helicase gene of the ORF1 (set forth in SEQ ID NO:3), and the complete 3' noncoding region (NCR).

EXAMPLE 8

Sequence Analysis of the ORF1 Region

The sequences of the three independent cDNA clones have the same size but differ in 16 nucleotide positions. However, at any given position, two of the three cDNA clones have the same nucleotide. Therefore, a consensus sequence was produced. The resulting consensus sequence of the 3' half genomic fragment of avian HEV is 3,931 nucleotides in length, excluding the poly (A) tract at the 3' end and the sequence of the 5' sense primer used for amplification. Sequence analysis revealed that the novel virus associated with HS in chickens is genetically related to human and swine HEV. Two complete ORFs (ORFs 2 and 3), and one incomplete ORF1 were identified in this genomic region.

The incomplete ORF1 sequence of avian HEV was aligned with the corresponding regions of human and swine HEV strains. Significant nucleotide and amino acid sequence identities were found in the ORF1 region between avian HEV and known HEV strains (Table 2, below). The avian HEV ORF1 region sequenced thus far contained the complete RdRp gene and a partial helicase gene. The RdRp gene of avian HEV encodes 483 amino acid residues and terminates at the stop codon of ORF1. A GDD motif (positions 343-345 in RdRp gene) that is believed to be critical for viral replication was identified (Figs. 2A-2B), and this motif was found in all RdRps (G. Kamer *et al.*, "Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses," Nucleic Acids Res. 12:7269-7282 (1984)). The RdRp gene of avian HEV is 4 amino acid residues shorter than that of known HEV strains (Figs. 2A-2B), and shared 47% to 50% amino acid and 52% to 53% nucleotide sequence

identity with that of known HEV strains (Table 2, below). The C-terminal 146 amino acid residues of the incomplete helicase gene of avian HEV shared approximately 57-60% nucleotide sequence and 58-60% amino acid sequence identities with the corresponding region of other HEV strains. The helicase gene of avian HEV is the most conserved region compared to known HEV strains. There is no deletion or insertion in this partial helicase gene region between avian HEV and other HEV strains. A 439 bp sequence of BLSV is available in the helicase gene region (C. J. Payne *et al.*, 1999, *supra*), and avian HEV shared 80% nucleotide sequence identity with BLSV in this region.

Sub. C7

Table 2. Pairwise comparison of the RNA-dependent RNA polymerase (RdRp) gene of the avian HEV with that of known HEV strains

	Avian HEV	Burma	D11092 China	D11093 China	HEV-T1 China	Hetian China	Hydarabad India	K52-87 China	Madras	Mexico	Myanmar	Nepal	Sar-55 Pakistan	Swine HEV USA	US1 USA	US2 USA	X98292 India
Avian HEV		53[a]	53	53	53	53	52	53	52	52	53	53	53	52	52	52	53
Burma	49		93	93	76	93	96	93	95	74	98	96	93	75	74	75	93
D11092 China	47	94		97	75	98	92	98	91	76	93	92	98	75	75	75	94
D11093 China	49	98	94		74	97	92	98	91	76	93	91	97	75	74	75	94
HEV-T1 China	50	86	82	86		75	75	75	74	73	75	76	75	76	75	75	75
Hetian China	49	98	93	98	86		92	98	90	74	93	92	98	75	74	75	94
Hydarabad India	49	97	93	97	85	97		92	94	76	96	95	92	75	74	74	92
K52-87 China	49	99	94	98	87	98	98		91	76	93	92	98	75	75	75	94
Madras India	47	95	90	94	82	94	94	95		75	94	95	91	74	73	74	91
Mexico	48	88	84	88	85	88	87	89	85		76	76	77	74	62	73	76
Myanmar	49	99	93	98	86	97	97	98	95	88		95	93	75	74	75	92
Nepal	49	98	93	98	86	98	97	98	94	88	98		92	75	75	75	92
Sar-55 Pakistan	49	99	94	98	87	98	98	99	95	89	98	98		75	75	75	94
Swine HEV USA	49	87	83	87	89	87	86	88	84	86	87	87	88		92	92	76
US1 USA	49	87	82	87	89	87	86	87	83	86	87	87	87	99		92	75
US2 USA	49	87	82	87	88	87	86	87	83	86	87	87	87	99	98		75
X98292 India	49	98	94	98	87	98	97	99	94	89	98	98	99	88	88	88	

[a]The values in the table are percentage identity of amino acids (lower left half) or nucleotides (upper right half).

EXAMPLE 9

Sequence Analysis of the ORFs 2 and 3

The ORF2 gene of avian HEV consists of 1,821 nucleotides with a coding capacity of 606 amino acids, about 60 amino acids shorter than that of other HEV strains. The ORF2 gene of avian HEV overlaps with ORF3 (Figs. 3A-3C), and terminates at stop codon UAA located 130 bases upstream the poly (A) tract. The predicted amino acid sequence of ORF2 contains a typical signal peptide at its N-terminus followed by a hydrophilic domain (Fig. 4). The sequence of the avian HEV signal peptide is distinct from that of known HEV strains (Figs. 5A-5C). However, it contains common signal peptide features that are necessary for the translocation of the peptide into endoplasmic reticulum: a positively charged amino acid (Arginine) at its N-terminus, a core of highly hydrophobic region (rich in Leucine residues) and a cleavage site (SRG-SQ) between position 19 and 20 (Figs. 5A-5C). Sequence analysis of the ORF2 revealed that the region between the signal peptide and the conserved tetrapeptide APLT (positions 108-111) is hypervariable, and 54 amino acid residues of avian HEV are deleted in this region (Figs. 5A-5C). Three putative N-linked glycosylation sites were identified in the ORF2 of avian HEV: NLS (pos. 255-257), NST (pos. 510-512) and NGS (pos. 522-524). Three N-linked glycosylation sites were also identified in known HEV strains but the locations are different from those of avian HEV. The first glycosylation site in known HEV strains is absent in avian HEV (Figs. 5A-5C), and the third glycosylation site in avian HEV is absent in the known HEV strains.

~~The ORF2 gene of known HEV strains varies slightly in size, ranging from 655 to 672 amino acid residues, but most strains have a ORF2 gene of 660 amino acid residues.~~ The ORF2 of avian HEV has 606 amino acid residues, which is 54 amino acids shorter than that of most known HEV strains. The deletions are largely due to the shift of the ORF2 start codon of avian HEV to 80 nucleotides downstream from that of known HEV strains (Figs. 3A-3C). The putative capsid gene (ORF2) of avian HEV shared only 42% to 44% amino acid sequence identity with that of known HEV strains (Table 3, below), when the major deletion at the N-terminus is taken into consideration. However, when the N-terminal deletion is not included in the comparison, avian HEV shared 48% to 49% ~~amino acid sequence identity with the corresponding region of other HEV strains.~~

Subc8

Multiple sequence alignment revealed that the normal start codon of the ORF3 gene in known HEV strains does not exist in avian HEV due to base substitutions (Figs. 3A-3C). Avian HEV utilizes the ORF2 start codon of other HEV strains for its ORF3, and consequently the ORF3 of avian HEV starts 41 nucleotides downstream from the start codon of known HEV strains (Figs. 3A-3C). Unlike known HEV strains, the ORF3 gene of avian HEV does not overlap with the ORF1 and locates 33 bases downstream from the ORF1 stop codon (Figs. 3A-3C). The ORF3 of avian HEV consists of 264 nucleotides with a coding capacity of 87 amino acid residues, which is 24 to 37 amino acid residues shorter than that of known HEV strains. Sequence analysis indicated that the ORF3 of avian HEV is very divergent compared to that of known HEV strains.

Table 3. Pairwise comparison of the putative capsid gene (ORF2) of avian HEV with that of known HEV strains

	Avian HEV[a]	Avian HEV[b]	Burma	D11092 China	D11093 China	HEV-T1 China	Hetian China	Hydarabad India	KS2-87 China	Madras	Mexico	Myanmar	Nepal	Sar-55 Pakistan	Swine HEV USA	US1 USA	US2 USA	Egypt93	Egypt94	Morroco	AKL90
Avian HEV[a]			47	47	47	44	47	47	47	47	45	47	47	47	46	45	46	47	47	48	47
Avian HEV[b]			51	51	51	48	51	51	51	51	49	51	51	51	50	49	50	51	51	51	51
Burma	44	49		94	93	77	94	96	94	96	80	97	98	93	79	79	79	91	90	89	97
D11092 China	44	49	99		97	77	98	93	98	93	81	93	93	98	80	79	79	91	91	90	93
D11093 China	44	49	98	98		77	97	93	98	93	80	93	93	97	79	78	79	91	91	90	93
HEV-T1 China	42	48	88	88	87		77	76	77	77	77	78	77	78	78	78	79	77	77	78	77
Hetian China	44	49	99	99	98	88		93	98	93	80	93	93	98	80	79	79	91	91	90	93
Hydarabad India	44	49	97	97	96	86	96		93	95	80	95	97	92	79	78	79	90	90	89	97
KS2-87 China	44	49	99	99	98	88	98	97		93	81	93	93	98	80	79	79	91	91	90	93
Madras India	44	49	99	99	98	88	98	96	98		80	96	96	92	97	97	97	90	90	90	96
Mexico	43	48	93	93	92	86	92	91	92	92		80	80	81	78	77	79	80	80	81	80
Myanmar	43	48	98	98	98	87	98	96	98	98	92		96	93	79	79	79	91	90	89	96
Nepal	44	49	98	98	98	87	98	96	98	98	92	98		93	79	79	79	90	90	90	97
Sar-55 Pakistan	44	49	99	99	98	88	99	97	99	99	93	98	98		80	79	79	91	91	91	93
Swine HEV USA	43	49	91	91	90	90	91	89	91	91	90	91	90	91		92	92	79	79	80	79
US1 USA	43	49	91	92	91	88	91	90	91	91	90	91	91	91	97		91	78	79	79	79
US2 USA	44	49	91	91	91	90	91	90	91	91	90	92	91	91	98	98		79	79	79	79
Egypt93	44	49	98	98	97	88	98	96	98	98	92	98	97	98	91	92	92		96	91	91
Egypt94	44	49	99	99	98	88	98	96	98	98	93	98	98	98	91	92	91	99		91	90
Morroco	44	49	99	99	98	88	98	97	98	98	93	98	98	99	91	91	91	98	99		90
AKL90	44	49	99	99	98	88	98	97	98	98	93	98	98	99	91	91	91	98	98	99	

The values in the table are percentage identity of amino acids (lower left half) or nucleotides (upper right half).

[a]Percentage identity when the major deletion at the N-terminal region of ORF2 is taken into consideration.

[b]Percentage identity when the major deletion is not included in the comparison.

EXAMPLE 10

Sequence Analysis of the 3' NCRs

Subc.9

The region between the stop codon of the ORF2 and the poly (A) tail of avian HEV, the 3' NCR, is 130 nucleotides. Sequence analysis revealed that the 3' NCR of avian HEV is the longest among all known HEV strains. The 3 NCRs of known HEV strains range from 65 to 74 nucleotides (Fig. 6). Multiple sequence alignment indicated that the 3' NCRs of HEV is highly variable, although a stretch of sequence immediately proceeding the poly (A) tract is relatively conserved (Fig. 6).

EXAMPLE 11

Identification of a Major Deletion in the ORFs 2 and 3 Overlapping Region of Avian HEV

Sequence analyses revealed a major deletion of 54 amino acid residues in avian HEV between the putative signal peptide and the conserved tetrapeptide APLT of the ORF2 (Figs. 5A-5C). To rule out the possibility of RT-PCR artifacts, a pair of avian HEV-specific primers flanking the deleted region was designed (Table 1, Figs. 3A-3C). The 3' antisense primer (RdelAHEV) located before the ORF3 stop codon of avian HEV, and the 5' sense primer (FdelAHEV) located within the C-terminal region of the ORF1. To minimize potential secondary structure problems, reverse transcription was performed at 60°C with a One Step RT-PCR Kit (Qiagen Inc., Valencia, CA). PCR was performed with 35 cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. In addition, PCR was also performed with shorter annealing time and higher denaturation temperature to avoid potential problems due to secondary structures. The PCR reaction consisted of an initial enzyme activation step at 95°C for 13 minutes, followed by 35 cycles of denaturation at 98°C for 20 seconds, annealing at 55°C for 5 seconds and extension at 73°C for 1 minute. It has been reported that formamide or DMSO could enhance the capability of PCR to amplify certain genomic regions of HEV (S. Yin *et al.*, "A new Chinese isolate of hepatitis E virus: comparison with strains recovered from different geographical regions," Virus Genes 9:23-32 (1994)). Therefore, a sufficient amount to make 5% (v/v) of formamide or DMSO was added in the PCR reactions. A PCR product of the same size (502 bp) as

observed in a conventional PCR is produced with various different RT-PCR parameters and conditions including the addition of 5% (v/v) of formamide or DMSO, the use of higher denaturation temperature and short annealing time, and the synthesis of cDNA at 60°C (Fig. 7). The deletion was further confirmed by directly sequencing the 502 bp PCR product.

EXAMPLE 12

Phylogenetic Evidence of Avian HEV as a New Genotype

Phylogenetic analyses based on three different genomic regions of HEV (a 439 bp of the helicase gene, a 196 bp of the RdRp gene, and a 148 bp of the ORF2 gene) identified at least 5 distinct genotypes of HEV (Fig. 8). The topology of the three trees based on different genomic regions is very similar. Similar phylogenetic trees were also produced with the complete RdRp and ORF2 genes of HEV strains in which their sequences are known. Most Asian strains of HEV are related to the prototype Burmese strain and clustered together, and these Burmese-like Asian strains of HEV represent genotype 1. The African strains of HEV (Egypt 93, Egypt 94 and Morroco) were related to, but distinct from, Burmese-like strains in the genotype 1. The limited sequences available for these African strains do not allow for a determination of whether they represent a distinct genotype or a subgenotype within the genotype 1. The single Mexican strain of HEV represents genotype 2. The genotype 3 of HEV consists of two U.S. strains of human HEV (US1, US2), a U.S. strain of swine HEV, a New Zealand strain of swine HEV, and several European strains of human HEV (Greek 1, Greek 2, Italy). The genotype 4 includes several strains of HEV identified from patients in China (HEV-T1, Ch-T11, Ch-T21, 93G) and Taiwan (TW7E, TW4E, TW8E). Avian HEV is the most divergent and represents the new genotype 5. Based on the limited sequence available for BLSV, it appears that the BLSV identified from chickens in Australia clustered with the genotype 5 of avian HEV, but the avian HEV retained significant differences in nucleotide sequence indicating that the avian HEV represents a new and distinct viral strain. Phylogenetic evidence that avian HEV is the most divergent strain of HEV identified thus far and represents a new genotype.

EXAMPLE 13

Isolation of Avian HEV in Embryonated Chicken Eggs

Others have failed to isolate the agent associated with HS syndrome in chicken embryos with conventional routes of egg inoculation (H. L. Shivaprasad *et al.*, "Necrohemorrhagic hepatitis in broiler breeders," Proc. Western Poult. Dis. Conf., p. 6, Sacramento, CA (1995)). Previous studies in pigs and primates showed that the I.V. route of inoculation is the most sensitive method to infect animals with the hepatitis E virus (HEV) (P.G. Kasorndorkbua *et al.*, "Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs," J. Virol. Methods, In Press (2001); P.G. Halbur *et al.*, "Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human," J. Clin. Microbiol. 39:918-923 (2001); X. J. Meng *et al.*, "Experimental infection of pigs with the newly identified swine hepatitis E virus (swine HEV), but not with human strains of HEV," Arch. Virol. 143:1405-1415 (1998); X. J. Meng *et al.*, "Genetic and experimental evidence for cross-species infection by the swine hepatitis E virus," J. Virol. 72:9714-9721 (1998)).

Surprisingly, the present attempt to isolate the agent associated with HS syndrome by I.V. inoculation of embryonated eggs was successful. A sample of bile collected from a 42-week-old Leghorn chicken with HS syndrome in California was used as the virus source (G. Haqshenas *et al.*, "Genetic identification and characterization of a novel virus related to the human hepatitis E virus from chickens with Hepatitis-Splenomegaly Syndrome in the United States," J. Gen. Virol. 82:2449-2462 (2001)). The undiluted positive bile contained about 10^7 genomic equivalents (GE) of avian HEV per ml measured by an avian HEV-specific semi-quantitative PCR (*id.*). Specific-pathogen-free (SPF) eggs were purchased at 1 day of embryonated age (Charles River SPAFAS, Inc. North Franklin, CT.). At 9 days of embryonated age, 40 eggs were I.V.-inoculated with 100 µl of a 10^{-4} dilution of the original positive bile, and 20 eggs remain uninoculated as controls. On the day of natural hatching (21 days of embryonated age), half of the inoculated embryos were sacrificed, and bile and samples of liver and spleen were harvested. The other half of the inoculated embryos were allowed to hatch, and most of the hatched chickens were necropsied at 2 to 3 days of age. Bile and liver collected from the necropsied embryos and chickens were tested positive for avian HEV RNA. The titer

of virus in the bile recovered from inoculated embryos was about 10^6 GE/ml, indicating that avian HEV replicates in embryonated chicken eggs. Four hatched chickens were monitored continuously. The hatched chickens seroconverted to anti-HEV, and avian HEV shed in feces. The feces collected from a hatched chicken at 8 days of age contain about 10^5 to 10^6 GE/ml of 10% fecal suspension, and this was the source of avian HEV for the subsequent animal studies.

EXAMPLE 14

Experimental Infection of Young SPF Chickens with Avian HEV

As a first step to determine if chickens can be infected experimentally with avian HEV, 12 SPF chickens of 3-to-6 days of age were I.V.-inoculated, each with about 2 x 10^4 GE/ml of avian HEV. Two uninoculated chickens were kept in the same cage with the inoculated ones as contact controls. Eight uninoculated chickens housed in a separate room served as negative controls. Fecal swabs were collected from all chickens every 3 days and tested for avian HEV RNA. Weekly sera from all chickens were tested for anti-HEV antibodies. Avian HEV RNA was detected in the feces of all inoculated chickens but not of the controls. Fecal shedding of avian HEV lasted about 2 to 3 weeks from 9 to 28 days post-inoculation (DPI). As expected with a fecal-orally transmitted virus, the two uninoculated contact control chickens (housed in the same cage with the inoculated ones) also became infected, and fecal virus shedding in the two contact control chickens started late from 18 to 35 DPI. Seroconversion to anti-HEV antibodies in inoculated chickens (but not in controls) occurred at about 32 to 38 DPI. Two infected and two control chickens were necropsied each at 25 and 35 DPI. The biles and feces of the necropsied chickens were positive for avian HEV RNA. There were no significant gross lesions in the infected young chickens. Microscopic liver lesions in infected chickens (but not in controls) were characterized by lymphoplasmacytic hepatitis with moderate to severe periportal, perivascular/vascular and occasional random foci of infiltration of lymphocytes mixed with a few plasma cells. The results demonstrate the successful reproduction of avian HEV infection in young chickens of 3-to-6 days of age but not the full-spectrum of HS syndrome.

EXAMPLE 15

Experimental Reproduction of Avian HEV Infection and HS Syndrome in Leghorn SPF Layer Chickens and Broiler Breeder Chickens

The failure to reproduce the full-spectrum of HS syndrome in young chickens is not surprising since, under field conditions, only broiler breeder and laying hens of 30-72 weeks of age developed HS syndrome (H. L. Shivaprasad *et al.*, "Necrohemorrhagic hepatitis in broiler breeders," Proc. Western Poult. Dis. Conf., p. 6, Sacramento, CA (1995); C. Riddell, "Hepatitis-splenomegaly syndrome," DISEASE OF POULTRY, p. 1041 (1997)); S. J. Ritchie *et al.*, "Hepatitis-splenomegaly" syndrome in commercial egg laying hens," Can. Vet. J. 32:500-501(1991)). Thus, two additional studies were performed to determine if avian HEV infection and HS syndrome could be experimentally reproduced in SPF layer chickens and broiler breeder chickens.

Layer chickens: Twenty (20) Leghorn SPF layer chickens of 60 weeks of age were purchased from Charles River SPAFAS, Inc. North Franklin, CT. Ten chickens were I.V.-inoculated each with 10^4 GE/ml of avian HEV, and housed in 5 isolators of 2 chickens each. Another 10 chickens, kept in 5 isolators in a separate room, were uninoculated as negative controls. Fecal swabs were collected from all chickens every 4 days. Avian HEV RNA was detected by RT-PCR from 8 to 27 DPIs in feces of infected chickens but not of controls. Sera were collected every 10 days, and seroconversion to anti-HEV antibodies occurred as early as 20 DPI. Two infected and two control chickens were necropsied each at 13, 17 and 21 DPIs. Avian HEV RNA was detected in the biles and feces of necropsied inoculated chickens but not of controls. Gross lesions characteristic of HS syndrome were observed in infected chickens, including hepatomegaly, subcapsular hemorrhages in livers (Fig. 18B) and pale foci on splenic capsular. Ovarian regression was also noticed in some infected chickens.

Significant microscopic lesions of liver and spleen consistent with HS syndrome were observed in infected SPF layer chickens. Livers from infected chickens had lymphoplasmacytic hepatitis with mild to moderate infiltration of lymphocytes in the periportal and perivascular regions (Fig. 19B). There were also foci of lymphocytes randomly scattered throughout the liver. A few focal hepatocellular necrosis with lymphocyte infiltration was also observed. Spleens from infected chickens had a mild

increase in mononuclear phagocytic system (MPS) cells. No significant gross or microscopic lesions were seen in control chickens.

Broiler breeder chickens: Six broiler breeder chickens of 64 weeks of age were I.V.-inoculated each with 10^4 GE/ml of avian HEV. Another 6 chickens were uninoculated as controls. Fecal swabs were collected every 4 days, and avian HEV RNA was detected in feces of all inoculated chickens from 12 to 27 DPI but not from controls. Sera were collected every 10 days and, like SPF layer chickens, seroconversion to anti-HEV antibodies also occurred in broiler breeder chickens as early as 20 DPI. Two infected and two control chickens were each necropsied at 14 and 21 DPI. Like layer chickens, the infected broiler breeders also had gross lesions consistent with HS syndrome including swollen liver and hemorrhages in the live and spleen. Microscopic liver lesions were characterized by lymphoplasmacytic hepatitis with infiltration of lymphocytes in the periportal and perivascular regions, and mild to severe vacuolation of most hepatocytes. Sections of spleens had a mild increase in MPS cells. No significant gross or microscopic lesions were observed in controls.

These two studies demonstrate the successful reproduction of avian HEV infection and HS syndrome with characteristic gross and microscopic lesions in SPF layers and broiler breeder chickens. Avian HEV with a sequence identical to the virus in the inoculum was re-isolated from experimentally infected chickens. Thus, avian HEV as a causative agent of HS syndrome in chickens is confirmed in accordance with Koch's germ theory of disease (Koch, R., 1876, Untersuchungen ueber Bakterien V. Die Aetiologie der Milzbrand-Krankheit, begruendent auf die Entwicklungsgeschichte des Bacillus Anthracis. Beitr. z. Biol. D. Pflanzen 2: 277-310, In Milestones in Microbiology: 1556 to 1940, translated and edited by Thomas D. Brock, ASM Press. 1998, p. 89).

EXAMPLE 16

Evaluation of Field Isolates of Avian HEV from Chickens with HS Syndrome

Strains of human and swine HEVs are genetically heterogenic. To determine the extent of heterogeneity among avian HEV isolates, the helicase gene region of 8 additional avian HEV isolates from chickens with HS syndrome from different geographic regions of the U.S. was amplified by RT-PCR and sequenced (Table 4,

below), showing that field isolates of avian HEV from chickens with HS syndrome are heterogeneic. Sequence and phylogenetic analyses revealed that, like swine and human HEVs, avian HEV isolates identified from different geographic regions of the United States are also heterogeneic (Fig. 20). Avian HEV isolates shared 79 to 96% nucleotide sequence identities with each other, 76-80% nucleotide sequence identities with BLSV and about 60% identities with swine and human HEVs (Table 4, below). The data also suggested that the BLS disease in Australian chickens and the HS syndrome in North American chickens are caused by a similar virus with about 76-80% sequence identities.

Table 4. Pairwise comparison of the nucleotide sequences of the helicase gene region of 8 field isolates of avian HEV (shown in boldface) identified from chickens with HS syndrome in the U.S. with that of other selected HEV strains

	2966G	0242	4449	4090	3690	3158.5	3077	9318B	aHEV	BLSV	T1	Mexico	US2	Swine	Sar-55	Flock location	Year Isol.
2966G		83	81	79	81	98	79	96	86	77	56	60	59	59	61	WI	2000
0242	83		88	86	83	83	86	83	80	79	56	59	59	60	59	CA	1994
4449	81	88		96	84	83	96	82	80	77	56	60	60	60	59	NY	2000
4090	79	86	96		83	80	94	81	79	76	56	60	59	59	59	East coast	2000
3690	81	83	84	83		81	84	80	80	80	57	60	59	60	59	CT	2000
3158.5	98	83	83	80	81		80	96	86	78	57	61	59	60	61	CA	1997
3077	79	86	96	94	84	80		81	79	77	56	60	60	60	59	CA	1993
9318B	96	83	82	81	80	96	81		88	78	57	60	59	59	60	Midwest	2000
aHEV *	86	80	80	79	80	86	79	88		77	57	61	57	58	60	CA	1993
BLSV†	77	79	77	76	80	78	77	78	77		56	59	60	60	59		
T1	56	56	56	56	57	57	56	57	57	56		73	75	75	76		
Mexico	60	59	60	60	60	61	60	60	61	59	73		72	75	78		
US2	59	59	60	59	59	59	60	59	57	60	75	72		91	75		
Swine‡	59	60	60	59	60	60	60	59	58	60	75	75	91		75		
Sar-55¶	61	59	59	59	59	61	59	60	60	59	76	78	75	75			

*aHEV, the prototype avian HEV. †BLSV, the causative agent of BLS disease in Australian chickens.
‡Swine, the prototype U.S. swine HEV. ¶Sar-55, the Pakistani strain of human HEV.

EXAMPLE 17

Expression and Purification of the Truncated ORF2 Capsid Protein of Avian HEV in a Bacterial Expression System

The truncated ORF2 protein of avian HEV containing the C-terminal 268 amino acid residues of ORF2 was expressed and characterized. The 804 bp sequence of the C-terminus of the avian HEV ORF2 was amplified with a set of avian HEV-specific primers: a sense primer (5'-GGGGGATCCAGTACATGTACGGCCGGCCTG-3', which corresponds to SEQ ID NO:10) with an introduced *Bam*HI site (underlined), and an antisense primer (5'-GGGGAATTCTTAGGGTGGTGAGGGGAATG-3', which corresponds to SEQ ID NO:11) with an introduced *Eco*RI site (underlined). The *Bam*HI and *Eco*RI sites were introduced at the 5' ends of the sense and antisense primers, respectively, to facilitate subsequent cloning steps. Proofreading *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used for PCR amplification of the fragment. The obtained PCR amplified fragment was purified and digested with *Bam*HI and *Eco*RI restriction enzymes and cloned into the pRSET-C expression vector (Clontech Laboratories, Inc., Palo Alta, CA). The truncated ORF2 gene was in-frame with the coding sequence of the Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA) located upstream of the multiple-cloning site of the expression vector. *E. coli* DH5α cells were transformed with the recombinant plasmids. The recombinant expression vector was isolated with a Qiagen Plasmid Mini Kit (Qiagen Inc., Valencia, CA), and confirmed by restriction enzyme digestions and DNA sequencing.

The recombinant plasmids were transformed into BL21(DE3)pLysS competent cells that have been engineered to produce T7 RNA polymerase. Expression of the fusion protein was driven by a T7 promoter sequence upstream of the Xpress™ epitope sequence (Invitrogen Corporation, Carlsbad, CA). By using pRSET-C vector, the recombinant fusion protein is tagged by six tandem histidine residues at the amino terminus (N-terminus) that have a high affinity for ProBond™ resin (Invitrogen Corporation, Carlsbad, CA). The bacterial cells were grown in SOB broth containing 50 μg/ml of ampicillin and 25 μg/ml of chloramphenicol. Expression of the fusion protein was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4-5 hrs at 37°C. The fusion protein was expressed in *E. coli* strain BL21(DE3)pLysS as

inclusion bodies. To confirm that the over-expressed protein contains Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA), the crude bacterial lysates separated on a 12% polyacrylamide gel containing 0.1% SDS and transferred onto a nitrocellulose membrane (Osmonics, Inc., Minnetonka, MN). The immobilized protein on the membrane was incubated with a with horseradish peroxidase (HRP)-conjugated monoclonal antibody, known to be against Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA) at 1:5,000 dilution. The immunocomplexes were detected using 4-chloro-1-naphthol (Sigma, St. Louis, MO).

From 50 ml of bacterial cultures, the fusion protein was purified by the use of ProBond™ Purification System (Invitrogen Corporation, Carlsbad, CA) based on the affinity of ProBond™ resin for His-tagged recombinant fusion protein. Bacterial cells were lysed with guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) and insoluble debris was clarified by centrifugation at 3,000 g for 10 minutes at 4° C. The supernatant was added to the resin pre-equilibrated with the binding buffer and gently agitated for 10 minutes at room temperature to allow the fusion protein to bind the resin. The protein-bound resin was serially washed six times with denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride) twice at each pH of 7.8, 6.0 and 5.3. The fusion protein was eluted in the elution buffer containing 8 M urea, 20 mM sodium phosphate and 500 mM sodium chloride (pH 4.0). The fractions containing the highest concentrations of protein were determined by the use of Bio-Rad protein assay reagent (BioRad, Carlsbad, CA). Five micrograms of the purified protein was analyzed by SDS-PAGE. The purified fusion protein hybridized with the MAb against Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA).

The nucleotide sequence of the insert was confirmed by automated cycle sequencing. The recombinant plasmid containing the truncated ORF2 gene of avian HEV was transformed into *E. coli* strain BL21(DE3)pLysS. Upon induction with IPTG, the truncated ORF2 capsid protein of avian HEV was expressed in this bacterial strain with a very high yield. The expressed protein was observed on the gel at the size of about 32 kD (Fig. 21A). Samples taken at different time points revealed that the maximum expression occurred at about 4 to 5 hrs after induction with IPTG (Fig. 21A).

Western blot analysis using a monoclonal antibody against Xpress™ epitope (Invitrogen Corporation, Carlsbad, CA) of the fusion protein confirmed the expression of the avian HEV ORF2 protein (Fig. 21B). Although the bacterial cells used in this study contain pLysS plasmid to minimize the background protein expressions, background expression was still observed. The fusion protein was expressed as inclusion bodies in the bacterial cells and was shown to be insoluble. The protein purification method was very efficient and about 6 mg of protein were obtained from 50 ml of the bacterial culture.

EXAMPLE 18

Evaluation of Antigenic Epitopes of Capsid Protein of Avian HEV, Human HEV, Swine HEV and Australian Chicken BLSV

In Western blot analysis, the purified truncated ORF2 protein of avian HEV reacted with the antiserum obtained from chickens experimentally infected with avian HEV but not with sera from normal control chickens. To prepare antiserum against avian HEV, specific-pathogen-free (SPF) chickens (SPAFAS Inc.) were inoculated intravenously with a diluted bile sample containing 10^3 GE/ml of avian HEV. The inoculated chickens excreted avian HEV in the feces and seroconverted to avian HEV antibodies. The convalescent sera collected at 30 days post inoculation were used as the avian HEV antiserum in this experiment. The antiserum against Sar-55 strain of human HEV was prepared by immunizing SPF pigs with baculovirus expressed and HPLC-purified capsid protein of the Sar-55 HEV. The antisera against swine HEV and US2 strain of human HEV were convalescent sera from pigs experimentally co-infected with these two HEV strains. The antiserum against Australia chicken BLSV was also kindly provided by Dr. Christine Payne (Murdoch University, Australia). The putative capsid protein of human HEV Sar-55 and swine HEV were expressed in baculovirus systems as described herein. The recombinant proteins were a gift from Drs Robert Purcell and Suzanne Emerson (NIH, Bethesda, MD). The HPLC-purified recombinant ORF2 capsid proteins of human HEV Sar-55 and swine HEV were used in this study.

Western blot analyses were used to determine if the truncated ORF2 protein of avian HEV shares antigenic epitopes with that of human HEV, swine HEV and BLSV. The purified recombinant truncated ORF2 protein (250 ng/lane) of avian HEV was

separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were cut into separate strips and then blocked in blocking solution (20 mM Tris-Cl, 500 mM NaCl, pH 7.5) containing 2% bovine serum albumin (BSA) for 1 hour. The strips were then incubated overnight at room temperature with 1:100 dilutions of antisera against avian HEV, swine HEV, human HEV and BLSV in Tris-buffered saline (20 mM Tris-Cl, 500 mM NaCl, pH 7.5) (TBS) containing 0.05% Tween® 20 (polysorbate 20, commercially available from Mallinckrodt Baker, Inc., Phillipsburg, NJ) (TBST) and 2% BSA. The original purified antibody against BLSV was diluted 1:1000 in TBST. Dilutions 1:100 of preinoculation swine sera were used as the negative controls. The strips were washed 2 times with TBST and once with TBS. Following 3 hrs incubation with HRP-conjugated goat anti-swine IgG (1:2000, Research Diagnostics Inc., Flanders, NJ) and HRP-conjugated rabbit anti-chicken IgY (1:2000, Sigma, St. Louis, MO), the strips were washed as described above and the immunocomplexes were detected using 4-chloro-1-naphthol.

To further confirm the cross-reactivity between avian, swine and human HEVs, approximately 250 ng of HPLC purified recombinant capsid proteins of swine HEV and Sar-55 human HEV were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The blot was incubated with antisera against avian HEV, swine HEV and human HEV. Serum dilutions, incubation and washing steps were carried out as described above. Anti-chicken IgY conjugated with HRP was used as the secondary antibody as described above.

The purified truncated ORF2 protein of avian HEV reacted strongly in Western blot analyses with convalescent sera from SPF chickens experimentally infected with avian HEV, HEV antibodies (antisera) raised against the capsid protein of Sar-55 human HEV and convalescent sera against the US2 strain of human HEV and swine HEV, and the antiserum against the Australian chicken BLSV (Figs. 22A and 22B). The purified truncated avian HEV ORF2 protein did not react with the preinoculation control chicken sera. Convalescent antisera from chickens experimentally infected with avian HEV reacted with the HPLC-purified recombinant ORF2 protein of Sar-55 human HEV. Swine HEV antiserum reacted strongly with the recombinant swine HEV ORF2 antigen. The US2 and Sar-55 human HEV antisera reacted with the recombinant swine HEV ORF2

capsid protein. The Sar-55 human HEV antiserum reacted strongly with Sar-55 ORF2 capsid antigen, but to a lesser extent with heterologous antisera against swine and avian HEVs (Figs. 22A and 22B). The reaction signals between avian HEV antiserum, Sar-55 human HEV and swine HEV ORF2 proteins were also strong. These results showed that avian HEV shares antigenic epitopes in its ORF2 capsid protein with swine and human HEVs as well as BLSV.

EXAMPLE 19

Cross-Reactivity of Avian HEV, Swine HEV and Human HEV Using ELISA

To assess the cross-reactivity of avian HEV, swine HEV and human HEV under a different condition than above study, this experiment was conducted. The ELISA plates (commercially available from Viral Antigens, Inc., Memphis, TN; BD Biosciences, Bedford, MA and others) were coated for 2 hrs with recombinant avian HEV, swine HEV and human HEV capsid antigens at 37°C. Each antigen was used at a concentration of 2 μg/ml of sodium carbonate buffer, pH 9.6. The potential non-specific binding sites were blocked with blocking solution (10% fetal bovine serum and 0.5% gelatin in washing buffer). The antisera, used in Western blot analyses, were diluted 1/200 in blocking solution. The preinoculation sera from a pig and a chicken were used as the negative controls. Following 30 minutes incubation at 37°C, the plates were washed 4 times with washing solution (PBS containing 0.05% Tween® 20 (polysorbate 20, commercially available from Mallinckrodt Baker, Inc., Phillipsburg, NJ), pH 7.4). The HRP-conjugated secondary antibodies were used as described for Western blot analysis. Following 30 minutes incubation at 37°C, the plates were washed as described above and the antigen-antibody complexes were detected using 2, 2'-Azino-bis (3-ethylbenthiazoline-6-sulfonic acid). After 10 minutes incubation at room temperature, the optical density (OD) was measured at 405 nm.

The cross-reactivity of avian HEV, swine HEV and human HEV were further confirmed using ELISA. As can be seen from Fig. 23, each antiserum strongly reacted with the corresponding antigen. The OD generated by interaction of avian HEV antiserum against recombinant antigens of Sar-55 human HEV strain and swine HEV was as high as 0.722 and 0.655, respectively, while the OD indicating non-specific binding of

preinoculation ("preimmune") chicken serum remained as low as 0.142 and 0.103, respectively. The OD values obtained from cross-reacting of avian HEV antigen to antiserum against Sar-55 human HEV was almost twice the OD recorded when the preinoculation pig serum was used. The OD obtained from reaction of US2 human HEV almost did not differ from the OD obtained for the preinoculation serum.

EXAMPLE 20

Computer Analysis of Amino Acid Sequences

The predicted amino acid sequences of the truncated ORF2 protein of avian, swine and human HEV strains were compared with MacVector® program (Oxford Molecular, Inc., Madison, WI). Hydropathy and antigenic plots of the amino acid sequences were determined according to Kyte-Doolittle (J. Kyte & R. F. Doolittle, "A simple method for displaying the hydropathic character of a protein," J. Mol. Biol. 157:105-32 (1982)) and Welling (Welling *et al.*, "Prediction of sequential antigenic regions in proteins," FEBS Letters 188:215-18 (1985)) methods using the MacVector® computer program (Oxford Molecular, Inc., Madison, WI).

Analyses of the predicted amino acid sequences of the entire ORF2 revealed that avian HEV shares only about 38% amino acid sequence identities with swine, US2 and Sar-55 HEV strains. Swine HEV ORF2 shared about 98% and 91% amino acid identities with US2 and Sar-55 HEV strains, respectively. The ORF2 of Sar-55 human HEV shared 91% amino acid sequence identity with the US2 strain of human HEV. Amino acid sequence alignment of the truncated ORF2 protein of avian HEV with the corresponding regions of swine HEV, Sar-55 human HEV and US2 human HEV also revealed that the most conserved region of the truncated 267 amino acid sequence is located at its N-terminus (Fig. 24) which contains hydrophilic amino acid residues (Figs. 25A-25D). By using the Welling method (Welling *et al.*, 1985, *supra*) to predict antigenic domains of the protein, three antigenic regions located at amino acids 460-490, 556-566 and 590-600 were also hydrophilic (Figs. 25A-25D).

In the foregoing, there has been provided a detailed description of particular embodiments of the present invention for the purpose of illustration and not limitation. It is to be understood that all other modifications, ramifications and equivalents obvious to those having skill in the art based on this disclosure are intended to be included within the scope of the invention as claimed.